1685

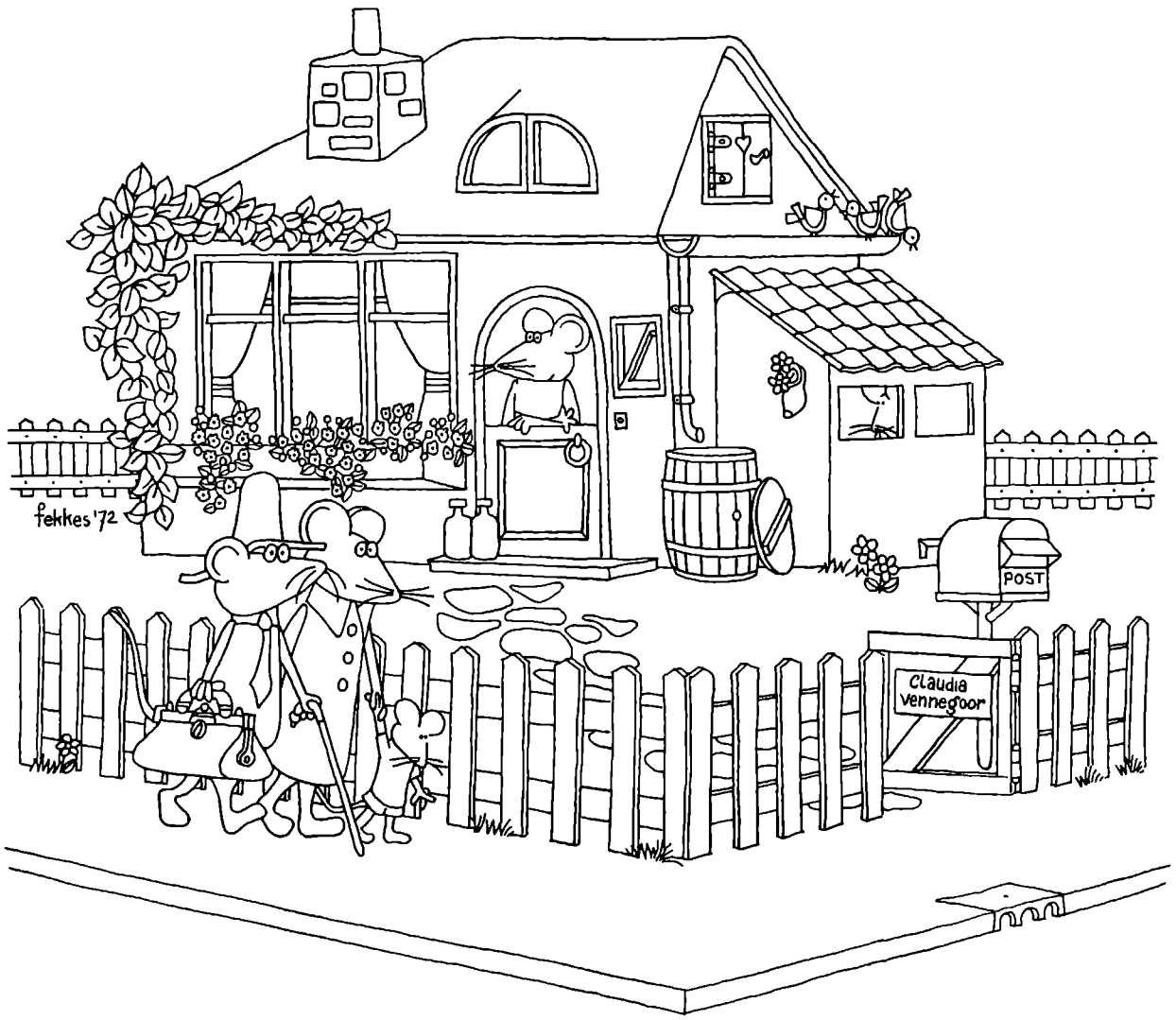
A Post-Microsomal Fraction from Rat Liver

isolation, characterization and function

C. Vennegoor

A Post-Microsomal Fraction from Rat Liver

isolation, characterization and function



PROMOTOR: PROF. DR. H. BLOEMENDAL

A POST-MICROSOMAL FRACTION FROM RAT LIVER
ISOLATION, CHARACTERIZATION AND FUNCTION

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de wiskunde en natuurwetenschappen aan de katholieke universiteit te Nijmegen, op gezag van de rector magnificus dr. G. Brenninkmeijer, hoogleraar in de faculteit der sociale wetenschappen, volgens besluit van de senaat in het openbaar te verdedigen op 15 juni 1972 des namiddags te 2 uur

door

Claudia Joanna Gerarda Maria Vennegoor

geboren te Didam

1972

MONDEEL-OFFSETDRUKKERIJ
AMSTERDAM

'.... it is no magic, that is a fiction of ignorance. There is no such thing as magic, though there is such a thing as a knowledge of the secrets of Nature.'

Sir Henry Haggard 'She'.

Aan mijn ouders

Dit proefschrift werd bewerkt op het biochemisch laboratorium van de Katholieke Universiteit van Nijmegen.

Een ieder die heeft bijgedragen tot dit resultaat wil ik hartelijk danken.

Een bijzonder woord van dank richt ik tot mej. J. van Westreenen, die talrijke isolaties voor mij heeft uigevoerd en mij bij de uitvoering van vele experimenten heeft geassisteerd.

Drs. A.L.H. Stols ben ik bijzonder dankbaar voor zijn aandeel in het electronenmicroscopisch gedeelte van het onderzoek.

De heer J. Reijnen dank ik hartelijk voor de bepalingen van sedimentatiewaarden in de analytische ultracentrifuge.

Professor dr. E.L. Benedetti (Institut de Biologie Moléculaire, Paris) en dr. W.S. Bont (Antoni van Leeuwenhoekhuis, Amsterdam) dank ik zeer voor hun discussies en critische opmerkingen.

Dr. M. Sluyser (Antoni van Leeuwenhoekhuis, Amsterdam) ben ik zeer erkentelijk voor de correctie van een groot deel van de tekst.

Een bijzonder woord van dank gaat uit naar de heer P. Spaan en medewerkers van het centraal dierenlaboratorium (hoofd dr. M. Dobbelaar), die voor mij de rattelevers hebben uitgeprepareerd.

Tenslotte dank ik de medewerkers van de afdeling medische tekenkamer (hoofd Chr. van Huyzen), medische fotografie (hoofd A. Reynen), glasinstrumentmakerij (hoofd L. Ramakers) en instrumentmakerij (hoofd K. Peters) voor hun snelle en vakkundige hulp.

Thanks are due to dr. C. Liébecq (editor in chief of the European Journal of Biochemistry) and Springer Verlag for their permission to publish this thesis in its present form.

CONTENTS

INTRODUCTION

In 1963 Hoagland and Askonas [1] reported that a post-microsomal fraction from rat liver stimulated the incorporation of amino acids into ribosomes *in vitro*. This fraction, which was named the X fraction by them, was isolated as a pellet by centrifuging the microsome-free supernatant of rat liver during 12 hours at 105 000 x *g*. When the pellet was resuspended in buffer and then added to an incubation mixture containing polyribosomes, amino acids, energy, cofactors, and the supernatant of the pellet as a source of soluble enzymes and tRNA, it enhanced the incorporation of amino acids into ribosomes. The X fraction was investigated by several authors, who attributed the stimulation of amino acid incorporation to different functions. They proposed that the X fraction contained messenger RNA [1], an aminoacyl-tRNA binding enzyme [2], and that it enhanced the interaction between ribosomes and messenger RNA [3, 4]. Since the data obtained were rather complex, and in some cases controversial, we decided to examine the X fraction in more detail. This thesis presents the results of our studies.

In order to familiarize the reader with a number of conceptions and reactions, the process of protein biosynthesis in the cytoplasm of eukaryotic cells is reviewed in chapter I.

In chapter II a survey is given of the literature on the action of the post-microsomal fraction (X fraction) on the amino acid incorporation into ribosomes *in vitro*. Some results of our own experiments are also included.

Chapter III and chapter IV are papers published by the author on this subject in the European Journal of Biochemistry.

REFERENCES

1. Hoagland, M.B. and Askonas, B.A., *Proc. Nat. Acad. Sci., U.S.A.* 49 (1963) 130.
2. Parthier, B., and Hultin, T., *Biochim. Biophys. Acta*, 149 (1967) 562.
3. Mizrahi, I.J., *Biochim. Biophys. Acta*, 108 (1965) 419.
4. Beard, N.S., and Armentrout, S.A., *Proc. Nat. Acad. Sci., U.S.A.* 58 (1967) 750.

CHAPTER I

The Process of Protein Synthesis

Protein biosynthesis occurs on polyribosomes, subcellular structures consisting of ribosomes and messenger RNA [1]. Here, tRNA-bound amino acids are polymerized to polypeptides, the order of amino acids being determined by the sequence of base triplets in the corresponding messenger RNA molecule (scheme 1).

(a) 1
(b) fMet – Ala –
(c) A–G–A–G–C–C–C–U–C–A–A–C–C–G- G–G–G–U–U–U–G–A–A–G–C–A–U–G–G–C–U–

5 10
Ser – Asn – Phe – Thr – Gln - Phe – Val – Leu – Val – Asn –
U–C–U–A–A–C–U–U–U–A–C–U–C–A–Gp

15 20
Asp – Gly – Gly – Thr – Gly – Asn – Val – Thr – Val – Ala –

25 30
Pro – Ser – Asn – Phe – Ala – Asn – Gly – Val – Ala – Glu –

35 40
Trp – Ile – Ser – Ser – Asn – Ser – Arg – Ser – Gln – Ala –
G–A–U–C–A–G–C–U–C–U–A–A–C–U–C–G–C–G–C–U–C–A–C–A–G–G–C–U–

45 50
Tyr – Lys – Val – Thr – Cys – Ser – Val – Arg – Gln – Ser –
U–A–C–A–A–A–G–U–A–A–C–C–U–G–U–A–G–C–G–U–U–C–G–U–C–A- G–A–G–C–

55 60
Ser – Ala – Gln – Asn – Arg – Lys – Tyr – Thr – Ile – Lys –
U–C–U–Gp G–C–A–A–A–U–A–C–A–C–C–A–U–U–A–A–A–

65 70
Val – Glu – Val – Pro – Lys – Val – Ala – Thr – Gln – Thr –
G–U–C–G–A–G–G–U–G–C–C–U–A–A–G–G–U–G–G–C–A–A–C–U–C–A–G–A–C–U–

75 80
Val – Gly – Gly – Val – Glu – Leu – Pro – Val – Ala – Ala –
G–U–U–G–G–U–G–G–U–G–U–A–Gp G–C–A–

85 90
Trp – Arg – Ser – Tyr – Leu – Asn – Met – Glu - Leu – Thr –
U–G–G–C–G–U–U–C–G–U–A–C–U–U–A–A–A–U–A–U–G–G–A–A–U–U–A–A–C–U–

95 100
Ile – Pro – Ile – Phe – Ala – Thr – Asn – Ser – Asp – Cys –
A–U–U–C–C–A–A–U–U–U–U–C–G–C–U–A–C–G–A–A–C–U–C–C–Gp

(continued on page 14)

105 110
Glu – Leu – Ile – Val – Lys – Ala – Met – Gln – Gly – Leu –

115 120
Leu – Lys – Asp – Gly – Asn – Pro – Ile – Pro – Ser – Ala –

125
Ile – Ala – Ala – Asn – Ser – Gly – Ile – Tyr

G–C–A–A–A–C–U–C C G–G–U–A–U–C–U–A–C–U–A–A–U–A–G–

A–U–G–C–C–G–G–C–C–A–U–U–C–A–A–A–C–A–U–G–A–G–G–A–U–U–A–C–C–C–

(d) fMet – Ser – Lys – Thr – Thr – Lys –
A–U–G–U–C–G–A–A–G–A–C–A–A–C–A–A–A–Gp

Scheme 1. *Amino acid sequence of the coat protein and nucleotide sequence found in the corresponding cistrons of bacteriophage R17 RNA* [2]. (a) Position of amino acid residues in coat protein; (b) amino acid sequence of coat protein; (c) nucleotide sequence in bacteriophage R17 RNA; (d) amino acid sequence of replicase.

1. ACTIVATION OF AMINO ACIDS

Before amino acids take part in polypeptide chain formation, they are activated in an enzymatic reaction in which the amino acid becomes attached to a transfer RNA molecule [3].

1.1. Reaction mechanism

The enzymes which catalyze the formation of aminoacyl-tRNA are the amino acid-RNA ligases (AMP) [E.C. 6.1.1 subgroup], more commonly named aminoacyl-tRNA synthetases or amino acid activating enzymes. The reaction takes place in two main steps [4].

[a] amino acid + ATP + enzyme $\rightleftharpoons$ aminoacyl~AMP-enzyme + P~P

[b] aminoacyl~AMP-enzyme + tRNA $\rightleftharpoons$ aminoacyl-tRNA + enzyme + AMP

It is generally believed that the carboxylic acid group of the amino acid is attached to the phosphate group of adenylic acid (reaction [a]) by high-energy bonding. The activated amino acid is subsequently transferred to the terminal ribose unit of the tRNA molecule [5-7] as is depicted in reaction [b]. Significant differences in behaviour observed during the purification of both aminoacyl-tRNA synthetases [8-10] and tRNA [11, 12] indicate the existence of individual enzymes and individual tRNA molecules for different amino acids.

Several aminoacyl-adenylate-enzyme complexes have been isolated upon incubation of ATP and magnesium ions with various amino acids and the appropriate aminoacyl-tRNA synthetases [7, 13-18]. This indicates that the formation of aminoacyl-tRNA proceeds through the two-step reaction shown above. It is likely that binding of ATP to the enzyme takes place before the binding of the amino acid during formation of the aminoacyl-adenylate intermediate [15-17, 19]. However, some authors believe that binding of the amino acid preceeds binding of ATP [14, 18], while there is also an indication that the order of addition of substrates to aminoacyl-tRNA synthetase can proceed by a random order of binding of ATP and amino acid [20]. When both substrates are bound to the amino acid-activating enzyme, pyrophosphate emerges from the complex [13, 14, 16-18]. After addition of tRNA to the aminoacyl-adenylate-enzyme complex, aminoacyl-tRNA is formed [7, 13-15, 18]. The formation of the aminoacyl-adenylate complex and of aminoacyl-tRNA can be reversed by addition of pyrophosphate [13, 15, 18] and AMP [7, 15] respectively.

The transfer of amino acids from enzyme-bound aminoacyl-adenylate to tRNA seems to be a more specific process than the formation of the

aminoacyl-adenylate-enzyme complex. For instance, if the pancreatic tryptophan-activating enzyme has formed a complex with the 'wrong' glycyl-adenylate, the glycine can only be attached to tRNA with low efficiency [7]. This finding may be compared with that obtained with the isoleucyl-tRNA synthetase from *Escherichia coli*. The latter enzyme can form a complex with either isoleucyl- or valyl-adenylate, but only the isoleucyl-adenylate complex reacts with tRNA. The valyl-adenylate complex breaks down in the presence of intact $tRNA^{Ile}$ [21]. This observation is in agreement with the assumption that tRNA specifically induces some conformational change in the aminoacyl-tRNA synthetase, enabling it to regulate the formation or stability of the aminoacyl-adenylate-enzyme complex [22].

Although it has clearly been demonstrated that the enzyme-bound aminoacyl-adenylate complex is able to transfer its activated amino acid to tRNA, some doubts have arisen as to whether this intermediate really exists under physiological conditions of aminoacyl-tRNA formation [23]. Experiments in which inhibitors are used suggest that the aminoacyl-adenylate complex is only formed in the absence of tRNA or hydroxylamine. In case of formation of aminoacyl-tRNA or aminoacyl-hydroxamate, the amino acid is probably directly transferred to tRNA or hydroxylamine respectively, with the simultaneous release of pyrophosphate [23].

It seems to be established that the stoichiometry of ATP and amino acid bound by the cognate enzyme is 1:1 [13-16, 18]. Beef pancreas tryptophanyl-tRNA synthetase, which has a molecular weight of 110 000 dalton [24, 25], can be dissociated into two equimolar components with different sedimentation values [18, 24]. The structure of the native enzyme is A_2B_2. At least 1.6 equivalents of tryptophanyl-adenylate, compared with 2.0 equivalents bound per molecule of native enzyme, can be transferred to tRNA in the absence of ATP [18]. This finding suggests that the dimers are also active. However, in seryl-tRNA synthetase from yeast (molecular weight 89 000 dalton [9]) only one molecule ATP and one molecule serine are bound per enzyme molecule [14]. It has been reported [26] that in phenylalanyl-tRNA synthetase from yeast (molecular weight 180 000 dalton [9]) also only one molecule $tRNA^{Phe}$ is combined with the tetrameric molecule of the enzyme.

1.2. Recognition of tRNA

Since the decoding of mRNA by aminoacyl-tRNA is independent of the amino acid attached to tRNA [27, 28], the mechanism by which tRNA is specifically recognized by its cognate synthetase is of special interest. It seems likely that the specificity of the enzyme for heterologous tRNA is very closely coupled to its specificity for the amino acid. Therefore, it is possible that a set of tRNA structures exists that is recognized by aminoacyl-tRNA synthetases from all organisms (see ref. 29) A completely intact structure of tRNA is not obligatory for recognition [29-34], but there are indications that at least parts of this structure, other than the specific recognition site, have to be intact in order to allow recognition of the tRNA molecule by the cognate synthetase [29, 34, 35]. The data obtained until now generally exclude the anticodon loop of tRNA as a specific recognition site for the aminoacyl-tRNA synthetases. It has tentatively been suggested that the first three base pairs of tRNA are part of the specific recognition site [29, 30]. Some data support a specific role of the acceptor stem in enzyme recognition [32, 36], but other data contradict this assumption [33, 37, see also ref. 31], or indicate that the acceptor stem is only a part of different recognition sites of tRNA [38]. It therefore seems possible that there is no universal tRNA site that is recognized by all synthetases or that recognition sites are scattered throughout the tRNA molecule [31].

1.3. Role of isoenzymes and isoacceptor tRNA molecules

Isoenzymes of aminoacyl-tRNA synthetases or isoacceptor tRNA's may play an important role in regulating protein synthesis. A selective synthesis of protein is generally assumed to be based either on the selective synthesis of mRNA or on a selective translation of the messages. Some cells might be unable to translate a given message, because of a lack of aminoacylated tRNA required to translate a specific triplet. A restricted amount of an aminoacyl-tRNA species can reflect changes of specific isoacceptor tRNA's. These changes have been observed to occur during differentiation in animal cells [39-43], in higher plants [44, 45] and in neoplastic cells [46-48]. Variations in the relative concentrations of amino acid-specific tRNA synthetases [44, 49-51] might also control the relative amount of free and aminoacylated forms of tRNA [50].

2. INITIATION OF PEPTIDE CHAIN FORMATION

A protein molecule cannot be synthesized unless an initiation complex has been formed. The mechanism of initiation in eukaryotes involves the binding of a small (40 S) ribosomal subunit and an initiator tRNA molecule to a specific initiator codon, usually AUG. This is followed by the addition of the large (60 S) ribosomal subunit. Protein factors and GTP are also required. The factors can be removed from the ribosomes by increasing the monovalent salt concentration [52-56]. The microsome-free supernatant from the brine shrimp [57] and from rat liver [58, 59], also contain components with characteristics of initiation factors.

2.1. Initiator tRNA

Initiation of protein synthesis at low concentration of magnesium ions (3 to 5 mM) requires a specific tRNA molecule, charged with methionine [60]. This initiator Met-tRNA molecule preferentially inserts the methionine into N-terminal position of the newly formed peptide chain [60-62]. It corresponds to the AUG or GUG codon at or near the 5' terminus of synthetic or native messenger RNA [63, 64]. Although the triplets AUG and GUG may code for methionine and valine respectively in internal position [60, 63], the presence of these codons at or near the 5' terminus of a mRNA seems to select the reading frame, whereas translation of other possible codons is severely suppressed [63].

Probably methionine is incorporated in N-terminal position in nearly all protein chains [61, 62, 65-69]. However, it is seldom detected as N-terminal amino acid, as it is split off from the growing peptide chain [63, 65-67], presumably by the action of a methionine amino-peptidase present in the ribosome [65].

In contrast with bacteria, in eukaryotic cytoplasm the initiator tRNA is not formylated when participating in the initiation of protein synthesis [60, 67, 70]. Yet the initiator tRNA shows some characteristics which discriminate this molecule from all other tRNA molecules. In the first place, the initiator Met-tRNA molecule derived from yeast [70, 71], mouse ascites cells [63, 72], rabbit liver [73] or calf lens [69] can be formylated by an enzyme (transformylase) present in *Escherichia coli*. In contrast, the initiator Met-tRNA from plant sources, *e.g.* embryos of wheat or horse bean, cannot enzymatically be transformylated [62, 67, 71, 74, 75]. In the second place, the eukaryotic initiator tRNA can be charged by *E. coli* synthetase [61, 62, 67, 72], whereas the tRNA molecule which inserts methionine only in internal positions cannot be charged. Thirdly, the peptide chain elongation factor transferase I fails to form a stable ternary complex with initiator Met-tRNA and GTP, indicating that the factor discriminates against this tRNA molecule [67, 71, 76].

2.2. Mechanism of initiation

The first step of initiation is binding of mRNA, which is probably present in the form of mRNP [77-82], to a native 40 S ribosomal subunit [83]. The 40 S subunit can eventually be attached to preexisting mono- or polyribosomes [78, 84-87], unless elongation of the peptide chain is inhibited [78]. The natural mRNA is specifically recognized by a protein factor (EF_3 or M_3) [53, 88]. This factor is also responsible for binding of mRNA to the 40 S subunit [53]. Binding of mRNA to EF_3 is enhanced by another protein factor (EF_1) [53].

The next step is binding of initiator Met-tRNA to the 40 S-mRNA complex. This step is dependent on GTP [56, 76, 83]. Hydrolysis of GTP occurs, but is not obligatory, indicating that in this step binding of GTP is sufficient [86]. The attachment of initiator Met-tRNA to the 40 S subunit requires the protein factors EF_1 and EF_2 (or M_1 and $M_{2(A,B)}$) and a low concentration of magnesium ions (3 to 5 mM) [56, 76, 86, 88].

Following upon the Met-tRNA binding to the 40 S subunit, the 60 S subunit must join the Met-tRNA-40 S complex in order to form the completed 80 S initiation complex [83, 86]. Only methionine which is bound to initiator tRNA can react with puromycin, suggesting that this Met-tRNA is bound at the peptidyl site* of the ribosome [62]. The formation of the 80 S ribosome-initiation complex is temperature sensitive, in contrast to the binding of mRNA [79]. Moreover, this step is accompanied by hydrolysis of GTP, which probably occurs between initiator tRNA binding and formation of a peptide bond with the second amino acid of the growing peptide chain [86].

When the 80 S-initiation complex has been formed, the aminoacyl-tRNA molecule which car-

* Each ribosome active in protein synthesis is supposed to bind two tRNA molecules, one at the aminoacyl site and the other at the peptidyl site [89].

ries the anticodon, corresponding with the next codon on the mRNA, can be attached to the amino acyl site of the ribosome. This occurs in the presence of the peptide chain elongation factor transferase I and GTP. A peptide bond is formed by the action of peptidyl transferase, which links the carboxylic acid group of the methionine residue of Met-tRNA present on the peptidyl site of the ribosome to the α-amino group of the amino acid residue of aminoacyl-tRNA located at the aminoacyl site. The dipeptide formed remains attached to the tRNA molecule on the aminoacyl site of the ribosome. In hemoglobin synthesis the formation of the first peptide bond is dependent on the initiation factor M_3 [56].

3. ELONGATION OF THE NASCENT PEPTIDE CHAIN

The dipeptide formed during initiation is extended in a process of peptide chain elongation in which the amino acids are polymerized sequentially. This process of peptide chain elongation is GTP-dependent and involves three different steps. Each of these is repeated during coupling of the next amino acid until the peptide chain is completed. The steps are: (a) Binding of aminoacyl-tRNA to the aminoacyl site of the ribosome. (b) Formation of a peptide bond by transfer of the nascent peptide chain from tRNA previously bound in the peptidyl site of the ribosome to the free α-amino group of the amino acid present as aminoacyl-tRNA at the aminoacyl site. (c) Translocation of the new peptidyl-tRNA to the peptidyl site. The latter step is accompanied by movement of the ribosome along the mRNA by one codon.

Two soluble proteins are required; one for binding of the newly incoming aminoacyl-tRNA and one for translocation of peptidyl-tRNA from the aminoacyl site to the peptidyl site of the ribosome [90-98]. The synthesis of the peptide bond is dependent upon ribosome-bound protein [99-101]. This is not removed by washing in a 0.5 M solution of ammonium chloride [101, 102].

3.1. Translocation of peptidyl-tRNA

After formation of the first peptide bond, the dipeptidyl-tRNA is present in the aminoacyl or acceptor site of the ribosome. Before the third amino acid can be incorporated into the growing peptide, the dipeptidyl-tRNA has to be transferred to the peptidyl or donor site of the ribosome [99, 101, 103-106]. The elongation factor transferase II catalyzes this process [99, 100]. This step is dependent on hydrolysis of GTP [100, 101, 103, 107].

Transferase II can be bound to the ribosome in the absence of translocation. GTP has to be present, but it can partially be replaced by the GTP-analog 5'-guanylyl methylene diphosphonate (GDPCP) [104, 108] or GDP [104], indicating that hydrolysis of GTP is not required for binding of the enzyme. Binding of transferase II to the ribosome requires both the 60 S and the 40 S subunit [109] and reduced sulphydryl groups on both the enzyme and the ribosome [110]. The binding occurs at the acceptor site of the ribosome, since peptidyl-tRNA at this location inhibits binding [110]. After dissociation of the ribosome, transferase II activity can specifically be demonstrated in the 60 S subunit [111].

3.2. Binding of aminoacyl-tRNA

When peptidyl-tRNA has been translocated on the ribosome, the aminoacyl site of the ribosome is available for binding a newly incoming aminoacyl-tRNA. This binding is dependent on mRNA [97, 101, 105, 106, 112] and occurs either in the absence of a protein factor at a moderately high concentration of magnesium ions (18 to 20 mM) [92, 101, 105, 106] or is catalyzed by the elongation factor transferase I at a lower magnesium ion concentration (6 to 12 mM) [92, 101, 105, 113]. The enzymatic binding of aminoacyl-tRNA is dependent on GTP [97, 105], which is not hydrolyzed in this part of the step [101, 103, 114]. However, GTP is specifically required [105, 106], probably because hydrolysis of GTP has to occur [107, 113] before synthesis of a peptide bond takes place [103, 106].

In the presence of GTP, transferase I is able to bind aminoacyl-tRNA in the absence of ribosomes [115, 116]. Probably the ternary complex of transferase I, aminoacyl-tRNA and GTP preceeds the binding to the ribosome, since this complex is bound more rapidly to the polyribosomes than are the separately added components [116]. Binding of transferase I to the ribosomes occurs on the 40 S subunit [109]. However, the 40 S subunit is able to bind aminoacyl-tRNA in the absence of GTP and transferase I. After addition of the 60 S subunit, the binding of aminoacyl-tRNA is stimulated by transferase I in the presence of GTP or GDPCP. The binding of tRNA is very likely stabilized by

interaction with the large subunit [101].

In the bacterial system the aminoacyl-tRNA binding factor can be separated into two fractions which are dependent on each other [117]. Mammalian transferase I can be separated into three different fractions. However, all fractions show biologically similar activities, although they differ in their apparent molecular weight [118]. It has therefore been suggested that mammalian transferase I is built up by three identical subunits [98].

3.3. Formation of the peptide bond

After binding of aminoacyl-tRNA in the aminoacyl site of the ribosome, a peptide bond is synthesized between the free α-amino group of aminoacyl-tRNA and the carboxylic acid redisue of the peptide chain which is linked to tRNA in the donor site. Deacylated tRNA is left at the donor site and leaves the ribosome. The peptide bond formation is catalyzed by the enzyme peptidyl transferase.

In contrast with the soluble elongation factors, the peptidyl transferase does not require GTP [97, 99, 100, 102, 106]. On rabbit reticulocyte ribosomes the peptidyl transferase shows two specific binding sites; a hydrophobic binding site for the attachment of aromatic amino acids, and a binding site for the 3'-penultimate CMP residue of aminoacyl-tRNA [119].

Up to now it is not clear whether one or more molecules of GTP are required in the cycle of reactions leading to peptide chain extension. The enzymes transferase I and transferase II are able to hydrolyze GTP independently from each other [92, 98, 120, 121]. However, the possibility remains that GTP, bound by transferase I, is hydrolyzed by transferase II [103].

4. TERMINATION OF THE COMPLETED PEPTIDE CHAIN

When the growing peptide chain has been completed, the chain cannot automatically leave the (poly)ribosome. A protein factor has been isolated from mammalian extracts which is specifically required for the release from mammalian ribosomes [122, 123]. In contrast with the multiple release components present in bacteria, namely the factors R_1, R_2 and S* [124-126], in mammalian systems the release factor seems to be either a complex of proteins or a multifunctional protein with characteristics of bacterial R and S factors [123]. Although up to now no release of a complete peptide chain has been studied in the animal system, the conditions which allow release of $fMet\text{-}tRNA_f$ from the complex formed from bacterial initiator tRNA and mammalian ribosomes closely resemble those found in the bacterial system.

In addition to the release factor mentioned, release in the animal system requires termination codon(s) and GTP [122]. Furthermore, peptidyl transferase activity might be required [122]. The termination codons are probably UAA, UGA and UAG [123]. Hydrolysis of GTP seems to be essential and occurs concomitant with, or after, binding of the release factor. It is, however, not directly coupled to peptidyl-tRNA hydrolysis, indicating that hydrolysis of GTP is an intermediate step in peptide chain termination [123]. Under influence of the release factor, the peptidyl transferase enzyme of the 60 S ribosomal subunit might cause hydrolysis of the peptidyl-tRNA instead of catalyzing the formation of an additional peptide bond [127].

5. DISSOCIATION OF THE RIBOSOMES

After release of the completed peptide chain, the ribosome probably leaves the mRNA and subsequently dissociates into subunits before participating again in initiation of protein synthesis. Although a dissociation factor has clearly been demonstrated in the bacterial system [128, 129], literature concerning a dissociation factor derived from eukaryotic cells is very scarce [130, 131]. In the yeast *Saccharomyces cerevisiae* a ribosomal dissociation factor has been described, which seems to be species specific [131]. The factor could only be demonstrated in the wash of native subunits. However, the occurrence of a dissociation factor in 80 S ribosomes is still questionable.

---

* R_1 recognizes the two termination codons UAA and UAG and R_2 recognizes UAA and UGA. Factor S stimulates the rate of release catalyzed by either factor R_1 or R_2.

REFERENCES

1. Wettstein, F.O., Staehelin, T., and Noll, H., *Nature*, 197 (1963) 430.
2. Sanger, F., *Biochem. J.* 124 (1971) 833.

3 Hoagland, M B , Stephenson, M L , Scott, J F , Hecht, L I , and Zamecnik, P C , *J Biol Chem* 231 (1958) 241

4 Stulberg, M P , and Novelli, G D , in *Methods in Enzymology*, vol 5, Academic Press, New York 1962

5 Novelli, G D , *Ann Rev Biochem* 36 (1967) 449

6 Kingdon, H S , Webster, L P , and Davie, E W , *Proc Nat Acad Sci , U S A* 44 (1958) 757

7 Wong, K K , and Moldave, K , *J Biol Chem* 235 (1960) 694

8 Alford, M A , Brotman, M , Chudy, M A , and Fraser, M J , *Can J Biochem Physiol* 41 (1963) 1135

9 Makman, M H , and Cantoni, G L , *Biochemistry*, 4 (1965) 1434

10 Christopher, C W , Jones, M E , and Stafford, D W , *Biochim Biophys Acta*, 228 (1971) 682

11 Gillam, I , Millward, S , Blew, D , von Tigerstrom, M , Wimmer, E , and Tener, G M , *Biochemistry*, 6 (1967) 3043

12 Nishimura, S , and Weinstein, I B , *Biochemistry*, 8 (1969) 832

13 Lagerkvist, U , Rymo, L , and Waldenstrom, J , *J Biol Chem* 241 (1966) 5391

14 Bluestein, H G , Allende, C C , Allende, J E , and Cantoni, G L , *J Biol Chem* 243 (1968) 4693

15 Allende, C C , Allende, J E , Gatica, M , Celis, J , Mora, G , and Matamala, M , *J Biol Chem* 241 (1966) 2245

16 Allende, C C , Chaimovich, H , Gatica, M , and Allende, J E , *J Biol Chem* 245 (1970) 93

17 Parin, A V , Savelyev, E P , and Kisselev, L L , *FEBS Letters*, 9 (1970) 163

18 Dorizzi, M , Labouesse, B , and Labouesse, J , *Eur J Biochem* 19 (1971) 563

19 Allende, J E , Allende, C C , Gatica, M , and Matamala, M , *Biochem Biophys Res Commun* 16 (1964) 342

20 Santi, D V , and Peña, V A , *FEBS Letters*, 13 (1971) 157

21 Baldwin, A N , and Berg, P , *J Biol Chem* 241 (1966) 839

22 Ohta, T , Shimada, I , and Imahori, K , *J Mol Biol* 26 (1967) 519

23 Loftfield, R B , and Eigner, E A , *J Biol Chem* 244 (1969) 1746

24 Preddi, E C , *J Biol Chem* 244 (1969) 3958

25 Lemaire, G , van Rapenbusch, R , Gros, C , and Labouesse, B , *Eur J Biochem* 10 (1969) 336

26 Befort, N , Fasiolo, F , Bollack, C , and Ebel, J -P , *Biochim Biophys Acta*, 217 (1970) 319

27 Chapeville, F , Lipmann, F , von Ehrenstein, G , Weisblum, B , Ray, W J , and Benzer, S , *Proc Nat Acad Sci , U S A* 48 (1962) 1086

28 von Ehrenstein, G , Weisblum, B , and Benzer, S , *Proc Nat Acad Sci , U S A* 49 (1963) 669

29 Chambers, R W , *Progress in Nucleic Acid Research and Molecular Biology*, vol 11, p 489, Academic Press, New York and London, 1971

30 Schulman, L H , and Chambers, R W , *Proc Nat Acad Sci , U S A* 61 (1968) 308

31 Yarus, M , *Ann Rev Biochem* 38 (1969) 841

32 Imura, N , Weiss, G B , and Chambers, R W , *Nature*, 222 (1969) 1147

33 Lagerkvist, U , and Rymo, L , *J Biol Chem* 245 (1970) 435

34 Kucan, Ž , Freude, K A , Kucan, I , and Chambers, R W , *Nature New Biology*, 232 (1971) 177

35 Streeck, R E , and Zachau, H G , *FEBS Letters*, 13 (1971) 329

36 Beltchev, B , and Grunberg Manago, M , *FEBS Letters*, 12 (1970) 27

37 Dudock, B , DiPeri, C , Scileppi, K , and Reszelbach, R , *Proc Nat Acad Sci , U S A* 68 (1971) 681

38 Mirzabekov, A D , Lastity, D , Levina, E S , and Bayev, A A , *Nature New Biology*, 229 (1971) 21

39 Holland, J J , Taylor, M W , and Buck, C A , *Proc Nat Acad Sci , U S A* 58 (1967) 2437

40 Lee, J C , and Ingram, V M , *Science*, 158 (1967) 1330

41 Yang, W K , and Novelli, G D , *Proc Nat Acad Sci , U S A* 59 (1968) 208

42 Garel, J P , Mandel, P , Chavancy, G , and Daillie, J , *FEBS Letters*, 7 (1970) 327

43 Garel, J P , Mandel, P , Chavancy, G , and Daillie, J , *FEBS Letters*, 12 (1971) 249

44 Anderson, M B , and Cherry, J H , *Proc Nat Acad Sci , U S A* 62 (1969) 202

45 Bick, M D , Liebke, H , Cherry, J H , and Strehler, B L , *Biochim Biophys Acta*, 204 (1970) 175

46 Taylor, M W , Buck, C A , Granger, G A , and Holland, J J , *J Mol Biol* 33 (1968) 809

47 Yang, W K , Hellman, A , Martin, D H , Hellman, K B , and Novelli, G D , *Proc Nat Acad. Sci , U S A* 64 (1969) 1411

48 Gallo, R C , and Pestka, S , *J Mol Biol* 52 (1970) 195

49 Strehler, B L , Hendley, D D , and Hirsch, G P , *Proc Nat Acad Sci , U S A* 57 (1967) 1751

50 Bick, M D , and Strehler, B L , *Proc Nat Acad Sci , U S A* 68 (1971) 224

51 Kanabus, J , and Cherry, J H , *Proc Nat Acad Sci , U S A* 68 (1971) 873

52 Naora, H , and Kodaira, K , *Biochim Biophys Acta*, 182 (1969) 469

53 Heywood, S M , *Proc Nat Acad Sci , U S A* 67 (1970) 1782

54 Shafritz, D A , Prichard, P M , Gilbert, J M , and Anderson, W F , *Biochem Biophys Res Commun* 38 (1970) 721

55 Naora, H , and Prichard, M J , *Biochim Biophys Acta*, 246 (1971) 269

56 Crystal, R G , Shafritz, D A , and Anderson, W F , *Proc Nat Acad Sci , U S A* 68 (1971) 1810

57 Zasloff, M , and Ochoa, S , *Proc Nat Acad Sci , U S A* 68 (1971) 3059

58 Leader, D P , Wool, I G , and Castles, J J , *Proc Nat Acad Sci , U S A* 67 (1970) 523

59 Wettenhall, R E H , Leader, D P , and Wool, I G , *Biochem Biophys. Res Commun* 43 (1971) 994

60 Smith, A E , and Marker, K A , *Nature* 226 (1970) 607

61 Bhaduri, S , Chatterjee, N K , Bose, K K , and Gupta, N K , *Biochem Biophys Res Commun* 40 (1970) 402

62 Yarwood, A , Boulter, D , and Yarwood, J N , *Biochem Biophys Res Commun* 44 (1971) 353

63 Brown, J C , and Smith, A E , *Nature*, 226 (1970) 610

64 Chatterjee, N K , Bose, K K , Woodley, C L , and Gupta, N K , *Biochem. Biophys Res Commun* 43 (1971) 771
65 Jackson, R , and Hunter, T , *Nature*, 227 (1970) 672
66 Wigle, D T , and Dixon, G H , *Nature*, 227 (1970) 676
67 Ghosh, K , Grishko, A , and Ghosh, H P , *Biochem. Biophys Res Commun* 42 (1971) 462
68 Caffier, H , Raskas, H J , Parsons, J T , and Green, M , *Nature New Biology*, 229 (1971) 239
69 Strous, G , van Westreenen, J , and Bloemendal, H , *FEBS Letters*, 19 (1971) 33
70 RajBhandari, U L , and Ghosh, H P , *J Biol Chem.* 244 (1969) 1104
71 Richter, D , Lipmann, F , Tarrago, A and Allende, J E , *Proc Nat Acad Sci , U S A* 68 (1971) 1805
72 Drews, J , Hogenauer, G , Unger, F , and Weil, R , *Biochem. Biophys Res Commun* 43 (1971) 905
73 Chatterjee, N K , Bose, K K , and Gupta, N K *Biochem. Biophys Res Commun* 40 (1970) 402
74 Leis, J P , and Keller, E B , *Biochem. Biophys Res Commun* 40 (1970) 416
75 Tarrago, A , Monasteiro, O , and Allende, J E , *Biochem. Biophys Res Commun* 41 (1970) 765
76 Shafritz, D A , and Anderson, W F , *Nature* 227 (1970) 918
77 Spohr, G , Granboulan, N , Morel, C , and Scherrer, K , *Eur J Biochem.* 17 (1970) 296
78 Lebleu, B , Marbaix, G , Werenne, J , Burny, A , and Huez, G , *Biochem. Biophys Res Commun* 40 (1970) 731
79 Lebleu, B , Marbaix, G , Huez, G , Temmerman, J , Burny, A , and Chantrenne, H , *Eur J Biochem.* 19 (1971) 264
80 Olsnes, S , *Eur J Biochem.* 23 (1971) 248
81 Olsnes, S , *Eur J Biochem.* 23 (1971) 557
82 Berns, A J M , de Abreu, R A , van Kraaikamp, M , Benedetti, E L , and Bloemendal, H , *FEBS Letters*, 18 (1971) 159
83 Heywood, S M *Nature*, 226 (1970) 696
84 Hoerz, W , and McCarty, K S , *Biochemistry*, 63 (1969) 1206
85 Hoerz, W , and McCarty, K S , *Biochim. Biophys Acta* 228 (1971) 526
86 Shafritz, D A , Laycock, D G , Crystal, R G and Anderson, W F , *Proc Nat Acad Sci , U S A* 68 (1971) 2246
87 Pragnell, I B , Marbaix, G , Arnstein, H R V , and Lebleu, B , *FEBS Letters*, 14 (1971) 289
88 Prichard, P M , Gilbert, J M , Shafritz, D A , and Anderson, W F , *Nature*, 226 (1970) 511
89 Warner, J R , and Rich, A , *Proc Nat Acad Sci , U S A* 51 (1964) 1134
90 Richter, D , and Lipmann, F , *Biochemistry*, 9 (1970) 5065
91 Yaniv, Z , and Staples, R C , *Biochim Biophys Acta*, 232 (1971) 717
92 Legocki, A , and Marcus, A , *J Biol Chem* 245 (1970) 2814
93 Pelly, J W , and Stafford, D W , *Biochim. Biophys Acta*, 204 (1970) 400
94 Gasior, E , and Moldave, K , *J Biol Chem.* 240 (1965) 3346
95 Klink, F , Kramer, G , Nour, A -M , and Petersen, K G , *Biochim. Biophys Acta*, 134 (1967) 360
96 Lamfrom, H , and Squires, R F , *Biochim. Biophys Acta*, 61 (1962) 421
97 Arlinghaus, R , Shaeffer, J , and Schweet, R , *Proc Nat Acad Sci , U S A* 51 (1964) 1291
98 McKeehan, W L , and Hardesty, B , *J Biol Chem* 244 (1969) 4330
99 Skogerson, L , and Moldave, K , *J Biol Chem.* 243 (1968) 5361
100 Siler, J , and Moldave, K , *Biochim Biophys Acta*, 195 (1969) 138
101 Busiello, E , di Girolamo, M , and Felicetti, L , *Biochim Biophys Acta*, 228 (1971) 289
102 Gatica, M , and Allende, J E , *Biochim Biophys Acta*, 228 (1971) 732
103 Skogerson, L , and Moldave, K , *Arch Biochem. Biophys* 125 (1968) 497
104 Skogerson, L , and Moldave, K , *J Biol Chem.* 243 (1968) 5354
105 Siler, J , and Moldave, K , *Biochim. Biophys Acta*, 195 (1969) 123
106 Siler, J , and Moldave, K , *Biochim. Biophys Acta*, 195 (1969) 130
107 Culp, W , McKeehan, W , and Hardesty, B , *Proc Nat Acad Sci , U S A* 64 (1969) 388
108 Skogerson, L , and Moldave, K , *Biochem. Biophys Res Commun.* 27 (1967) 568
109 Rao, P , and Moldave, K , *J Mol Biol* 46 (1969) 447
110 Baligala, B S , and Munro, H N , *Nature New Biology*, 233 (1971) 257
111 Traugh, J A , and Collier, R J , *FEBS Letters*, 14 (1971) 285
112 Arlinghaus, R , Favelukes G , and Schweet, R , *Biochem. Biophys Res Commun* 11 (1963) 92
113 Lin, S Y , McKeehan, W L , Culp, W , and Hardesty, B , *J Biol Chem.* 244 (1969) 4340
114 Ibuki, F , and Moldave, K , *J Biol Chem.* 243 (1968) 44
115 Rao, P , and Moldave, K , *Biochem. Biophys Res Commun* 28 (1967) 909
116 Richter, D , *Biochem. Biophys Res Commun* 38 (1970) 864
117 Seeds, N W , and Conway, T W , *Biochem. Biophys. Res Commun* 28 (1967) 1047
118 Schneir, M , and Moldave, K , *Biochim. Biophys Acta*, 166 (1968) 58
119 Harris, R J , Hanlon, J E , and Symons, R H , *Biochim Biophys Acta*, 240 (1971) 244
120 Felicetti, L , and Lipmann, F , *Arch Biochem. Biophys* 125 (1968) 548
121 McKeehan, W , Sepulveda, P , Lin, S -Y , and Hardesty, B , *Biochem. Biophys Res Commun* 34 (1969) 668
122 Goldstein, J L , Beaudet, A L , and Caskey, C T , *Proc Nat Acad Sci , U S A* 67 (1970) 99
123 Beaudet, A L , and Caskey, C T , *Proc Nat Acad Sci , U S A* 68 (1971) 619
124 Gupta, N K *J Biol Chem.* 243 (1968) 4959
125 Milman, G , Goldstein, J , Scolnick, E , and Caskey, T , *Proc Nat Acad Sci , U S A* 61 (1968) 768
126 Goldstein, J , Milman, G , Scolnick, E , and Caskey, T , *Proc Nat Acad Sci , U S A* 65 (1970) 430
127 Caskey, C T , Beaudet, A L , Skolnick, E M , and Rosman, M , *Proc Nat Acad Sci , U S A* 68 (1971) 3163

128 Subramanian, A R , Ron, E Z , and Davies, B D , *Proc Nat Acad Sci , USA* 61 (1968) 761
129 Bade, E G , Gonzalez, N S , and Algranati, I D , *Proc Nat Acad Sci , USA* 64 (1969) 654
130 Favelukes, G , Sorgentini, D , Bard, E , and Martone, C , in a 'free communication' on the 8th International Congress of Biochemistry, Montreux, 1970
131 Pêtre, J , *Eur J Biochem.* 14 (1970) 399

CHAPTER II

A Post-Microsomal Fraction and its Role in the Incorporation of Amino Acids *in vitro*

Ten years ago, Hoagland [1] and Hoagland and Askonas [2] described a post-microsomal fraction from rat liver which appeared to play a role in the incorporation of amino acids in a cell-free system. This fraction was isolated from the microsome-free supernatant by centrifugation during 12-13 h at 105 000 x *g*. The final pellet was designated as X fraction. After resuspending, the X fraction stimulated the incorporation of amino acids when a pH 5 fraction of the supernatant of the X fraction (which differs from the usual pH 5 fraction in that it lacks X) was used as source of 'soluble enzymes'. The X fraction consisted of protein and RNA. Most of the RNA appeared to be tRNA, which itself did not cause the observed stimulation of amino acid incorporation. Since the total RNA isolated from the X fraction stimulated the amino acid incorporation, and since this RNA was labelled more rapidly than that in the microsomal fraction or in the pH 5 fraction lacking X, Hoagland and Askonas concluded that they were dealing with messenger-like RNA. However, total X fraction stimulated the incorporation of amino acids to a higher level than did total RNA isolated from the X fraction. Therefore, the authors concluded that the RNA was bound to protein which protected the nucleic acid against degradation by ribonuclease.

In 1965 Mizrahi extended these studies [3]. His results, however, failed to indicate a messenger RNA function. He reasoned that if the effect of the X fraction were due to its content of mRNA, the incorporation of [^{14}C]phenylalanine in the presence of both polyuridylic acid and the X fraction could not be higher than that obtained when polyuridylic acid and the X fraction were added separately. However, he found that the incorporation of [^{14}C]phenylalanine was highly stimulated by the X fraction in a cell-free system containing polyuridylic acid, preincubated ribosomes, and soluble enzymes in which X fraction was lacking. In the system containing non-preincubated polyribosomes, and hence containing endogenous mRNA, the incorporation of [^{14}C]leucine was not affected by adding polyuridylic acid. When, however, also X fraction was added, the incorporation of leucine was slightly decreased. These results suggested that free ribosomes released from the polyribosomes during protein synthesis had been bound to polyuridylic acid instead of to the endogenous mRNA. The author concluded that the X fraction might initiate and/or stabilize ribosome-mRNA complexes which were active in protein synthesis.

Beard and Armentrout drew a similar conclusion concerning the action of an X fraction isolated from rabbit reticulocytes [4]. These authors also detected an enhanced incorporation of phenylalanine directed by polyuridylic acid when the X fraction was added. Moreover, when X fraction plus polyuridylic acid were added to a cell-free system which had ceased synthesizing protein, amino acid incorporation was resumed, whereas separate addition of X fraction or polyuridylic acid had no effect.

The possibility of a messenger RNA function of the X fraction was reinvestigated by Parthier and Hultin in 1967 [5]. After adding rapidly labelled RNA from the X fraction to an amino acid incorporating system, these authors detected a low degree of stimulation, but only when tested in the cell-free system of *Escherichia coli*. They also investigated the amino acid-activating enzymes and transferases. The amino acid-activating enzymes were not found to be selectively accumulated in the X fraction, except leucyl-tRNA synthetase. The activity of this enzyme was higher in the X fraction than in the pH 5 fraction lacking X. The authors demonstrated that the transfer activity was predominantly present in the X fraction. Therefore, they suggested that its action could be explained by accumulation of transferases in the X fraction.

The effect of the X fraction on incorporation of amino acids in a cell-free system thus seemed to be rather complex. Since its action was interpreted on the basis of different processes, we examined the effect of this fraction in more detail [6-8].

In preliminary experiments we found that the polyuridylic acid-dependent system could not easily be saturated with the supernatant of the X fraction or with a pH 5 fraction lacking X. In contrast, when the endogenous system was investi-

gated, saturation with the supernatant of X could be demonstrated Addition of X fraction stimulated the incorporation of amino acids Likewise, addition of X fraction to the polyuridylic acid-dependent system containing non-saturating amounts of either the supernatant of X or the unfractionated microsome-free supernatant, stimulated polyphenylalanine synthesis However, when the latter system was saturated with the supernatant of X or with the unfractionated microsome-free supernatant respectively, addition of X fraction did not affect phenylalanine incorporation

RNA, extracted from the X fraction by treatment with phenol-dodecyl sulphate, did not stimulate the incorporation of amino acids by preincubated ribosomes to a significant extent, when compared with the effects of 28 S or 18 S ribosomal RNA Purified tRNA did not significantly stimulate the incorporation of phenylalanine in the polyuridylic acid-dependent system, while the amino acid incorporation in the endogenous system was inhibited by tRNA

Activity of the X fraction was fully preserved after chromatography on DEAE-Sephadex A-50 Its main activity eluted at a 0 18 M concentration of potassium chloride This fraction appeared to be free of the aminoacyl-tRNA binding factor transferase I Moreover, when partially purified transferase I was added to the endogenous system saturated with the supernatant of X, no stimulation of incorporation of amino acids was detected These experiments and those described elsewhere [6], showed that the effect of the X fraction could not be explained by transferase I activity

In contrast with the conclusions of other authors, we proposed that amino acid-activating enzymes played an important role in the action of the X fraction The main results of our investigations are described in the two papers presented in chapters III and IV

REFERENCES

1 Hoagland, M B , *Cold Spring Harbor Symp Quant Biol* 26 (1961) 153
2 Hoagland, M B , and Askonas, B A , *Proc Nat Acad Sci U S A* 49 (1963) 130
3 Mizrahi, I J , *Biochim Biophys Acta*, 108 (1965) 419
4 Beard, N S , and Armentrout, S A , *Proc Nat Acad Sci , U S A* 58 (1967) 750
5 Parthier B , and Hultin, T , *Biochim Biophys Acta*, 149 (1967) 562
6 Vennegoor, C , and Bloemendal, H , *Eur J Biochem* 15 (1970) 161
7 Vennegoor, C J G M , Stols, A L H , and Bloemendal, H , *J Mol Biol* 65 (1972) 375
8 Vennegoor, C , and Bloemendal, H , *Eur J Biochem* 26 (1972) 462

Eur. J. Biochem. 15 (1970) 161—170

Effect of a Purified Post-Microsomal Fraction on Amino Acid Incorporation *in vitro*

Claudia VENNEGOOR and Hans BLOEMENDAL

Laboratorium voor Biochemie, Universiteit van Nijmegen

(Received February 11/May 6, 1970)

A protein fraction (Fraction X) was isolated from rat liver cell sap from which ribosomes had been removed previously by centrifugation for 14.5—15 h at 105000 × *g*. Fraction X sedimented at approx. 28 S. The isolated fraction restored amino acid incorporation into protein *in vitro* to values obtained when total cell sap was used as an enzyme source. The active fraction was purified by precipitation with ammonium sulphate and by gel filtration on Sephadex G-200. This purification resulted in removal of the ribonuclease inhibitor which was present in the crude Fraction X. The result of each purification step was examined by electrophoresis on polyacrylamide gels. Evidence is provided that the activity of fraction X cannot be ascribed to ribosomal subunits, but is mainly due to the action of synthetases.

Several years ago Hoagland [1,2] described a post-microsomal fraction from rat liver which appeared to play a role in the incorporation of amino acids into protein, by a cell-free system containing microsomes. The effect of this so-called Fraction X was tentatively attributed to mRNA activity. Mizrahi [3] who reinvestigated this problem suggested that Fraction X might initiate and/or stabilize the interaction between free ribosomes and mRNA. Parthier and Hultin [4] concluded that the main effect was due to aminoacyl transferase activity. The possibility that a minor part of Fraction X consisted of mRNA was also envisaged by these authors. These contradictory assumptions led us to investigate Fraction X from rat liver in more detail. We were able to achieve separation of Fraction X from the polysome-stabilizing principle [5] which is indentical to the ribonuclease inhibitor [6].

EXPERIMENTAL PROCEDURES

Materials

GTP and pyruvate kinase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany); phosphoenolpyruvate (sodium salt) and ATP from Sigma Chemical Co., (St. Louis, Mo., U.S.A.). Polyuridylic acid ($s_{20} = 5.37$ S) was supplied by Miles Chemical Co. (Elkhart, Indiana, U.S.A.). L-[^{14}C]-Phenylalanine (495 mC/mmole), DL-[^{14}C]leucine (55.2 mC/mmole) and algal hydrolysate (52 mC/mmole) were purchased from the Radiochemical Centre (Amersham, England).

Unusual Abbreviations. Messenger RNA, mRNA; transfer RNA, tRNA; polyuridylic acid, poly U.

Preparation of Rat Liver Polysomes

Medium B consisted of 50 mM Tris adjusted to pH 7.6 with a solution containing 4 N HCl, 25 mM KCl, 10 mM magnesium acetate and 0.35 M sucrose. This medium was used when the polysomes were prepared for the endogenous incorporation system. Medium B′ differed from Medium B in its magnesium acetate concentration which was 5 mM. Medium B′ was used when the polysomes were prepared for the poly U-dependent incorporation of polyphenylalanine.

Two- or three-month old male Wistar albino rats were starved overnight. After decapitation, the livers were collected in Medium B or B′, weighed and homogenized in 2.5 ml medium/g wet tissue-weight. A Teflon glass homogenizer cooled to 0° was used. Five strokes at 500 rev./min were applied. Polysomes were prepared from the homogenate as described by Bloemendal *et al.* [7].

The discontinuous gradients were prepared in the same medium as those in which the rat liver homogenates were prepared. Centrifugation was carried out at 75000 × *g* (on average) for 14—16 h in rotor 30 of the Spinco model L preparative ultracentrifuge. The pellets were resuspended in Medium B or B′. RNA concentration was measured at 260 nm, assuming that an absorbance of 25 corresponded to 1 mg RNA/ml. The ribosomes were used immediately or were frozen and kept at −20° at a concentration of 2 mg RNA/ml when they were used for endogenous incorporations, or 1 mg RNA/ml when they were used for poly U-dependent incorporations. The latter batches were subsequently diluted with medium B′ to the proper concentration before being used for incorporation experiments.

Preparation of Total Cell Sap, Supernatant X and Fraction X

For the preparation of total cell sap, a $15000 \times g$ supernatant fraction was prepared in the same manner as when polyribosomes were prepared. However, the livers were collected and homogenized in Medium B′ to which 5 mM β-mercaptoethanol or 2mM dithiothreitol had been added. The supernatant was centrifuged for 75 min at $105000 \times g$ in a Ti50 rotor of the Spinco model L preparative ultracentrifuge. The fatty layer on the top of the preparation was removed by aspiration, and the upper three quarters of the supernatant were collected. This fraction will be denoted as total cell sap.

Supernatant X and Fraction X were prepared from total cell sap by further centrifugation during 14.5—15 h in a Ti50 rotor at $105000 \times g$ according to the procedure of Hoagland [1,2]. The supernatant fluid (designated Supernatant X) was separated from the brownish-red translucent pellet by aspiration. By means of a manually operated Teflon glass homogenizer, the pellet was suspended in 2 ml Medium B′ containing either mercaptoethanol or dithiothreitol. The homogenate was centrifuged at $4500 \times g$ for 10 min in a Martin Christ junior centrifuge. The supernate is designated Fraction X according to Mizrahi [3] and Parthier and Hultin [4]. Total cell sap, Supernatant X and Fraction X were frozen in small portions and kept at — 20°.

Precipitation with Ammonium Sulphate

Fractionation of proteins by precipitation with ammonium sulphate was carried out by the following procedure. Fraction X (180—300 mg protein) was suspended in 12—20 ml Medium B′ containing mercaptoethanol or dithiothreitol. Then a Tris-Cl solution (pH 7.6, 2 M) was added to a final concentration of 0.1 M Tris. After stirring, a solution of saturated ammonium sulphate (3.9 M) was added dropwise for 30 min until 30 % saturation was achieved. Tris (2 M) was added to a final concentration of 0.1 M, and then mercaptoethanol was added to a final concentration of 5 mM. Fraction X was continually stirred at 0°. After addition of the last drop of the ammonium sulphate solution, stirring was continued for an additional 60 min. The suspension was then centrifuged for 30 min at $15000 \times g$. The precipitate (0—30 % saturation of ammonium sulphate) was suspended in 1 ml of Medium B′ containing mercaptoethanol or dithiothreitol. The brown supernatant was saturated with the ammonium sulphate solution to 60 % saturation, under continuous stirring at 0°. The mixture was made 0.1 M with respect to Tris (pH 7.6), and 5 mM with respect to mercaptoethanol. Stirring was continued for an additional 2 h. The suspension was centrifuged and the precipitate (30—60 % saturation of ammonium sulphate) suspended in 1 ml of Medium B′ containing mercaptoethanol or dithiothreitol. Solid ammonium sulphate was added slowly to the red supernatant to 90 % saturation (44.9 g/100 ml solution). Stirring was continued for an additional 2—4 h. After centrifugation, Medium B′ containing mercaptoethanol or dithiothreitol was added until the precipitate (60—90 % saturation of ammonium sulphate) was dissolved. The supernatant which was practically colourless, was discarded.

All protein fractions were freed from ammonium sulphate by gel filtration on a column of Sephadex G-25 (coarse; column size 60 cm × 1.5) which had been equilibrated with sucrose-free Medium B′ containing mercaptoethanol or dithiothreitol. Fractions of 0.5 ml were collected in the region of the protein peak. Ammonium sulphate content was assayed with a solution of 1 M $BaCl_2$. The protein fractions which had been freed from ammonium sulphate were finally concentrated by the addition of dry Sephadex G-25 (coarse) and subsequent elution with sucrose-free Medium B′ containing mercaptoethanol or dithiothreitol. When the fractions were not used immediately, Medium B′ containing mercaptoethanol or dithiothreitol and 2 M sucrose was added to a final concentration of 0.35 M sucrose. The fractions were then frozen and kept at — 20°.

Gel Filtration on Sephadex G-200

Gel filtration was carried out on a column (column size 44 × 2.5 cm) of Sephadex G-200 which had been equilibrated with sucrose-free Medium B′ containing mercaptoethanol or dithiothreitol. The protein moiety of Fraction X which precipitated between 30 and 60 % saturation of ammonium sulphate, was freed from ammonium sulphate as described above. 4 ml, containing 80—90 mg protein, were layered. Fractions of 60 drops were collected. The active fractions were concentrated with Sephadex G-25 (coarse) as described above.

Chromatography on DEAE-Sephadex

Chromatography on a column of DEAE-Sephadex A-50 (column size 1 × 35 cm) was used for separating tRNA from other components. The crude Fraction X (150—200 mg protein, dissolved in 10—15 ml) was applied to a column which had been equilibrated with sucrose-free Medium B′ containing mercaptoethanol. The adsorbed proteins were washed with 150—200 ml of this medium. Then a linear salt gradient was applied. The mixing chamber contained 250 ml Medium B′ (which contained mercaptoethanol), and the reservoir 250 ml NaCl in Medium B′ (which contained mercaptoethanol). Fractions of 5 ml were collected. The active fraction was eluted between 0 and 0.25 M NaCl. Transfer RNA was eluted with 0.5 M NaCl.

Sucrose Density Gradient Centrifugation

Both the crude Fraction X and the active fractions obtained after gel filtration on Sephadex G-200 were submitted to further purification by centrifugation on sucrose gradients. Linear 10—35 % sucrose gradients in Medium B′ containing dithiothreitol were used. The gradients were centrifuged for 12 h at 25000 rev./min in a Spinco SW 25I rotor of the Spinco model L preparative ultracentrifuge. After centrifugation, the tubes were punctured and fractions of 15 drops each collected. The absorbance was measured at 260 nm and 280 nm. The active fractions were concentrated by vacuum dialysis.

Incorporation of [^{14}C]Leucine in the Endogenous System

The incubation mixtures contained 50 mM Tris-Cl pH 7.6, 70 mM KCl, 8 mM magnesium acetate, 0.5 mM ATP, 0.25 mM GTP, 5.0 mM phosphopyruvate, 25 μg/ml pyruvate kinase, a mixture of 19 unlabelled amino acids (each in a concentration of 60 μM and 30 μM DL-[^{14}C]leucine). Final volume 0.5 ml. Total cell sap or Supernatant X and Fraction X proteins were added as indicated in the results section. Each incubation vessel contained 200 μg polysomal RNA. Incubation was carried out for 30 min at 37°.

After incubation the tubes were chilled in ice for 15 min. Correction was made by adding to each tube the proper amount of protein. The tubes were shaken and 0.5 ml of a 10 % (w/v) solution of trichloroacetic acid was added. After shaking again, 5 ml of a 5 % (w/v) solution of trichloroacetic acid were added. The tubes were kept at 0° for 15 min. After centrifugation the precipitate was suspended in 5 ml of 5 % (w/v) trichloroacetic acid, heated for 30 min at 90° and poured on Millipore filter (25 mm; pore size 0.45 μm). The filters were washed with cold trichloroacetic acid. After drying at 70° for 10 min, the radioactivity of the samples was either measured on a thin window gas flow counter (Nuclear Chicago) with an efficiency of 30 %, or the filter was placed in 10 ml of a scintillation system of toluene containing 0.3 % 2,5-diphenyloxazole and 0.02 % 1,4-bis-(5-phenyloxazolyl-2)-benzene. In the latter case radioactivity was measured in the Packard Tri-carb liquid scintillation spectrophotometer model 4322 with an efficiency of 85 %.

Except for the experiments with the Sephadex G-200 eluate and the sucrose density gradient centrifugation, all assays were performed in duplicate.

Incorporation of [^{14}C]Phenylalanine in the Poly U-Dependent System

The preincubation mixtures (Bont *et al.* [8]) contained 50 mM Tris-Cl pH 7.6, 25 mM KCl, 8 mM magnesium acetate, 0.625 mM ATP, 0.313 mM GTP, 6.25 mM phosphopyruvate, 31.25 μg/ml pyruvate kinase and a mixture of 20 unlabelled amino acids (including phenylalanine), each in a concentration of 125 μM. Final volume 0.4 ml. Total cell sap or Supernatant X protein were added as indicated. The preincubation mixture contained 25 μg polysomal RNA, unless indicated otherwise. Preincubation was carried out for 30 min at 37° followed by cooling in ice for at least 15 min. Incubation was then carried out, after adding to the preincubation mixture: 250 nC [^{14}C]phenylalanine, 100 μg poly U and various amounts of Fraction X protein. Final volume 0.5 ml. Salts were added to a final concentration of 50 mM Tris-Cl pH 7.6, 70 mM KCl and 8 mM magnesium acetate. Incubation was performed for 30 min at 37°. Then the mixtures were treated as described above.

Except for the experiments with the Sephadex G-200 eluate all assays were performed in duplicate.

Transferase Activity

Assays for transfer factors and the preparation of transfer factor T_I were essentially carried out as described by Schneir and Moldave [9]. Transfer factor T_{II} was obtained by extraction of rat liver microsomes with deoxycholate [10]. The polysomes were prepared from the microsomal fraction by extraction with deoxycholate and further purified from contaminating transfer factors by centrifugation through discontinuous sucrose gradients containing 0.5 M ammonium chloride [11]. The freshly prepared transfer factor T_{II} was activated [12] by preincubation for 5 min at 37° with polysomes (25 μg RNA), 0.2 mM GTP, 2 mM dithiothreitol, 80 mM ammonium chloride, 6 mM magnesium acetate and 60 mM Tris-Cl buffer pH 7.6 in a total volume of 0.4 ml. Then aminoacyl-tRNA (labelled with [^{14}C]leucine), transfer factor T_I and/or purified Fraction X were added. Tris-Cl, ammonium chloride, magnesium acetate, GTP and dithiothreitol were brought to the same concentration as in the preincubation mixture. Final volume 1 ml. After incubation for 30 min at 37°, the mixtures were treated as described above.

The assays were performed in duplicate.

Isolation of tRNA from Rat Liver

Transfer RNA was isolated from rat liver according to the method of Brunngraber [13], with slight modifications. The tRNA was stripped by incubation for 90 min at 37° in 1.8 M Tris-Cl of pH 8.0.

Labelling of Rat Liver tRNA

The tRNA was charged with [^{14}C]leucine and 19 other non-radioactive amino acids, as described by Lapidot *et al.* [14] for the labelling of tRNA with phenylalanine. Cell sap from rat liver which had

been freed from tRNA by chromatography on DEAE-Sephadex A-50 [15] was used as source of synthetases.

Preparation of RNA from Polysomes and Fraction X

RNA was extracted from polysomes according to the method of Konings and Bloemendal [16]. However, phenol extraction was carried out at 4° for 30 min.

RNA from the crude and purified Fraction X was prepared by extraction with phenol containing sodium dodecyl sulphate [17]. The extractions were carried out for 30 min at 37°.

Electrophoresis on Polyacrylamide Gels

Separation of proteins by electrophoresis on polyacrylamide gels was performed as described by Bloemendal [18]. Separation of RNA by polyacrylamide gel electrophoresis was performed according to the method of Loening [19] as modified by Konings and Bloemendal [16]. Proteins were stained with Amido Black 10 B in methanol—acetic acid—H_2O (50:7:43, by volume). RNA was stained with a solution containing 0.2% toluidin blue O and 10% acetic acid. Lipoid components were stained beforehand for 45 min at room temperature with Sudan Black B in ethylene glycol according to the method of McDonald and Ribeiro [20], or stained after the electrophoretic run with oil red O in methanol—acetic acid—H_2O (60:10:30, by volume) [21]. Excess stain was removed electrophoretically with a 2% acetic acid solution.

Protein Determination

Protein was determined by the method of Lowry *et al.* [22], using bovine serum albumin as standard. The standards and blanks contained the same concentration of mercaptoethanol or dithiothreitol as did the protein samples.

RESULTS AND DISCUSSION

The Assay System

The incorporation of either [^{14}C]leucine or [^{14}C]-phenylalanine, the latter in the presence and absence of poly U, was examined with increasing amounts of total cell sap and Supernatant X fraction. The results are shown in Fig. 1. In the endogenous system (leucine incorporation) a plateau was reached by the addition of approximately 0.4 mg of Supernatant X protein to 200 μg of polysomes, whereas in the poly U-dependent system a plateau could only be detected if a very small amount of polysomes was present (Fig. 2). This observation clearly shows that the optimal incorporation conditions determined for the leucine incorporation may not be adopted automatically for the phenylalanine-poly U system as was done by Mizrahi [3].

Effect of the Crude Fraction X on the Endogenous System

In Table 1 the stimulatory activity of the crude Fraction X in the presence of total cell sap is compared to the effect in the presence of Supernatant X. Although the effect with total cell sap is only small it can be concluded that it is deficient in some activity present in Fraction X. In the presence of Supernatant X the requirement for Fraction X is more pronounced. Moreover the incorporation of [^{14}C]leucine in the presence of Supernatant X can equalize the level obtained with total cell sap, provided optimal quantities of crude Fraction X are added to the incubation mixture.

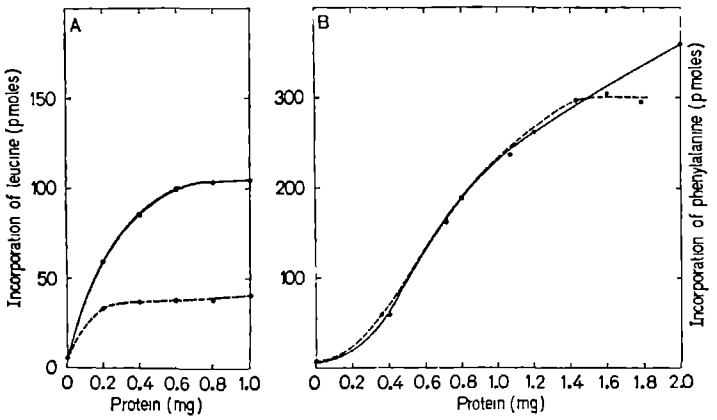


Fig. 1. *Effect of soluble proteins on the incorporation of amino acids* in vitro. (A) Incorporation of [^{14}C]leucine into rat liver polysomes (200 μg). (B) Incorporation of [^{14}C]phenylalanine in the poly U-dependent system; each preincubation mixture contained 25 μg of polysomes; in the absence of poly U, 9.2 pmoles were incorporated. The incubations were carried out as described under Experimental Procedures. Soluble proteins from total cell sap (●——●) and from Supernatant X (●----●)

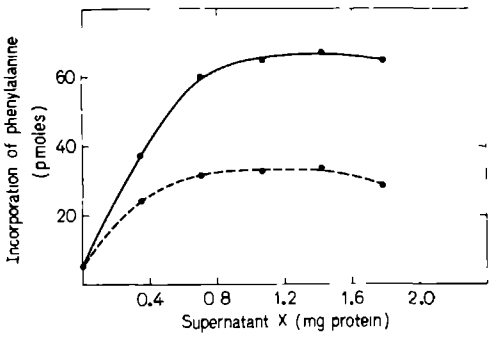


Fig 2 *Incorporation of [^{14}C]phenylalanine in the poly U-dependent system* The preincubation mixtures contained 5 µg (●----●) or 10 µg (●——●) of polysomes

Table 1 *Stimulation by crude Fraction X of [^{14}C]leucine incorporation into protein in the cell free system of rat liver* The incubation mixture contained 0 8 mg protein of total cell sap or of Supernatant X Fraction

Fraction X	Incorporation in the presence of Total cell sap			Supernatant X		
mg protein	pmoles		%	pmoles		%
0	98	97	100	36	38	100
0 6	115	114	117	79	76	210
1 2	110	116	116	90	100	257
1 8	113	122	121	105	102	280
2 4	118	112	117	107	104	285
3 0	110	103	109	100	103	274
3 0[a]	2	2		3	2	
3 0[b]	3	2		3	3	

[a] In the absence of polysomes
[b] Incubation at 0°

Partial Purification of Fraction X

Precipitation with Ammonium Sulphate Ammonium sulphate precipitation alone did not adequately cause fractionation of Fraction X activity In the presence of Supernatant X, stimulation of leucine incorporation was observed by the addition of the proteins which precipitated at 30% saturation with ammonium sulphate, as well as by the addition of those which precipitated between 30 and 60% saturation or between 60 and 90% saturation The proteins, however, which precipitated between 30 and 60% saturation with ammonium sulphate showed the largest specific activity (Table 2)

Gel Filtration on Sephadex G 200 The gel filtration pattern observed with the protein moiety of Fraction X which precipitated between 30 and 60% saturation with ammonium sulphate is shown in Fig 3 In Fig 3A fractions 14—58 are examined for stimulatory activity on the incorporation of leucine in the endogenous system Fig 3B shows the stimulatory effect of corresponding fractions in the poly U-dependent incorporation of phenylalanine The fractions

Table 2 *Stimulation by the ammonium sulphate precipitate of Fraction X of the [^{14}C]leucine incorporation into protein in the cell free system of rat liver*
The incubation mixture contained 0 8 mg protein of Supernatant X and 0 375 mg of Fraction X

Addition	Leucine incorporation		
	pmoles		%
None	38	38	100
Crude Fraction X	72	72	190
0—30% sat $(NH_4)_2SO_4$	48	—	126
30—60% sat $(NH_4)_2SO_4$	79	76	204
60—90% sat $(NH_4)_2SO_4$	55	54	143

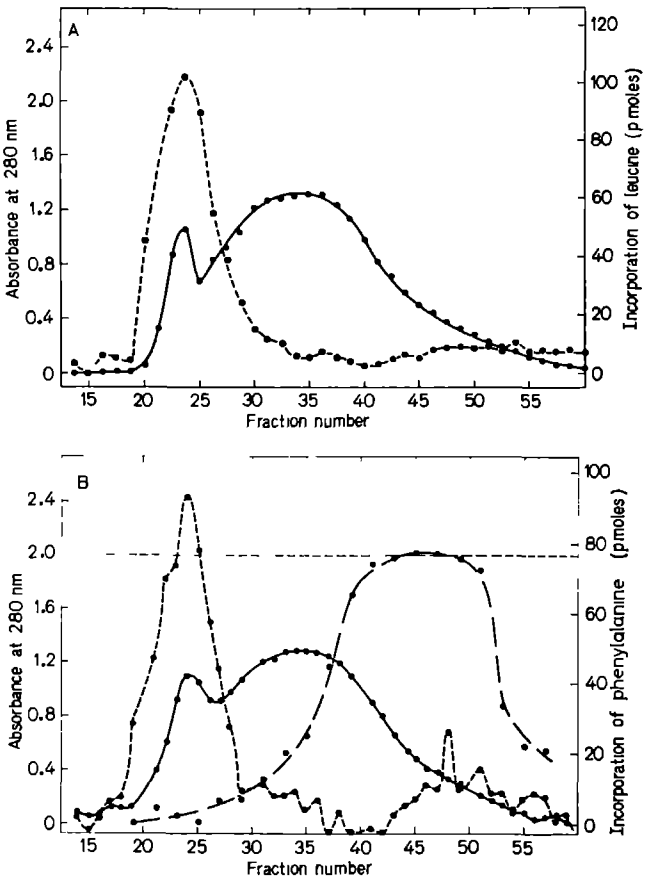


Fig 3 *Elution from Sephadex G 200 of the proteins of Fraction X precipitated between 30 and 60% saturation with ammonium sulphate* (A) [^{14}C]Leucine incorporation in the endogenous system (B) [^{14}C]Phenylalanine incorporation in the poly U dependent system The incubation mixtures (0 5 ml) for the endogenous amino acid incorporation contained each 0 4 mg of Supernatant X protein and 150 µl of the eluted fractions For the poly U dependent system the incubation mixtures (0 5 ml) consisted of 0 2 ml of preincubation mixture (containing 25 µg of polyribosomes and 0 9 mg of Supernatant X protein), 100 µg of poly U, 250 nC of [^{14}C]phenylalanine and 200 µl of the eluated fractions Corrections were made for salts, amino acids, ATP, GTP, phosphopyruvate and pyruvate kinase Fractions appearing within the void volume are not shown ●——●, Absorbance at 280 nm; ●----●, incorporation of [^{14}C]leucine or [^{14}C]phenylalanine, ●- - -●, ribonuclease inhibitor activity, ----, 100% inhibition

tion which stimulated amino acid incorporation emerged directly after the void volume of the Sephadex G-200 column, indicating that the active fraction probably had a molecular weight of 800000 or higher, provided it had globular structure. These results differ from the results reported by Beard and Armentrout [23]. In their studies, filtration on Sephadex G-200 of Fraction X prepared from rabbit reticulocytes caused the active component to emerge after the ribonucleoprotein particles. The latter migrated together to form a peak which was not retarded on the column. Fig. 3B also shows the elution pattern in which ribonuclease inhibitor activity was demonstrated. It has previously been shown that the inhibitor has a protecting effect on polysomes and might play a role in protein biosynthesis [6]. The activity corresponding to Fraction X appears to be completely separated from the ribonuclease inhibitor activity. This observation provides evidence that the effect of Fraction X may not be ascribed to inhibition of ribonuclease activity. The latter possibility could not be excluded in the earlier investigations [1—4, 23].

A small stimulatory effect on phenylalanine incorporation was consistently found in fractions 43 to 58. This may be due to the presence of a very small amount of tRNA in Fraction X (compare Fig. 8).

In the endogenous system the active fraction obtained after gel filtration showed a considerable stimulation on leucine incorporation when saturating quantities of Supernatant X were used during incubation. Also in incubations with saturating amounts of total cell sap, a slight stimulation by this fraction was found (Table 3, compare also Table 1). The stimulatory fraction kept its activity during more than 4 months when stored at $-20°$ after concentrating it with Sephadex G-25 and adding a 2 M sucrose solution (in Medium B′ containing dithiothreitol) to a final concentration of 0.35 M sucrose.

In the purified fraction, transferase I activity could be demonstrated whereas transferase II appeared to be absent (Table 4). On the other hand there was still an additional activity in Fraction X which could not be ascribed to the action of transferase I. This could be shown by adding a transferase I preparation, purified on hydroxylapatite and Sephadex G-200, to an incubation mixture in which Supernatant X was used as enzyme source. In this case no stimulation of the amino acid incorporation could be observed (Table 5). Likewise, preparations containing both transferase I and transferase II which were obtained by adsorbing the pH 5 supernatant of rat liver on hydroxylapatite, followed by washing the latter with Tris buffer and extracting it with 0.175 M and 0.25 M phosphate buffer, pH 6.8 [13], did not have stimulatory effect. Therefore we conclude that the transfer factors were not responsible for the action of Fraction X.

Table 3. *Stimulation by a partially purified Fraction X of the [^{14}C]leucine incorporation into protein in the cell-free system of rat liver*

Crude Fraction X had been precipitated with ammonium sulphate (30–60% saturation), freed from ammonium sulphate by gel filtration on Sephadex G-25 (coarse) and subjected to filtration on Sephadex G-200; 200 µg protein of the active fraction were added to the incubation mixture which contained 0.7 mg protein of total cell sap or 0.8 mg protein of Supernatant X

Conditions	Addition of active fraction from gel filtration on Sephadex G-200	Leucine incorporation		
		pmoles		%
Total cell sap	−	151	151	100
	+	181	183	121
Supernatant X	−	52	50	100
	+	176	170	339

Table 4. *Transferase activity of Fraction X*

Crude Fraction X was purified as described in Table 3. Transfer factors T_I and T_{II} and polysomes were prepared as indicated in Experimental Procedures, under transferase activity. Each incubation vessel contained 25 µg of tRNA acylated with [^{14}C]leucine (2800 counts/min) and 19 other unlabelled amino acids, 25 µg of polysomal RNA (126 µg protein) and saturating amounts of transfer factors and/or purified Fraction X as indicated

Transfer factor		Purified Fraction X	Leucine incorporation	
T_I	T_{II}			
µg	µg	µg	pmoles	
50	—	—	0.37	0.43
—	80	—	0.02	0.03
—	—	120	0.30	0.27
50	80	—	1.04	0.99
50	—	120	0.34	0.39
—	80	120	1.03	1.11
50	80	120	1.11	1.02
—	—	—	0.02	0.00

From Table 5 it appears that synthetase activity plays a major role in the effect of Fraction X. This was also confirmed by another experiment with purified Fraction X (Table 6). This observation is in sharp contrast to a statement by Mizrahi [3] that synthetase activity is not involved in the action of Fraction X. Parthier and Hultin [4], however, claim that at least leucyl-tRNA synthetase is concentrated in Fraction X, but according to the authors this activity probably does not constitute the basis of the stimulatory effect.

Sedimentation Analysis

The results obtained by gel filtration on Sephadex G-200 indicated that the active component of Frac-

Table 5. *Effect of purified Fraction X and of the purified transfer factor T_I on the incorporation of amino acids (A) and on the transfer of aminoacyl-tRNA into the material precipitated by hot trichloroacetic acid (B)*

Each incubation vessel contained unwashed free polysomes (25 μg RNA), Supernatant X (200 μg protein) and purified Fraction X and factor T_I as indicated. In the type A experiments, DL-[^{14}C]leucine, 19 unlabelled amino acids and an energy source were present in concentrations indicated under Experimental Procedures. In the type B experiments, conditions were the same as in type A experiments with addition of 50 μg aminoacyl-tRNA acylated with [^{14}C]leucine (4568 counts/min) and 19 other unlabelled amino acids, and omission of free amino acids, ATP, phosphopyruvate and pyruvate kinase

Experiment	Fraction X	Factor T_I	Leucine incorporation	
	μg	μg	pmoles	
Type A	—	—	6	6
	50	—	24	24
	100	—	27	26
	—	50	6	6
	—	100	6	6
	100	100	29	29
Type B	—	—	2	2
	50	—	3	4
	100	—	3	3
	—	50	3	3
	—	100	3	3
	100	100	3	3

Table 6. *Synthetase activity in the purified Fraction X*

Each incubation vessel contained, unless indicated otherwise, the purified Fraction X from Sephadex G-200 (120 μg protein), tRNA (stripped), ATP, 3 mM dithiothreitol, 50 mM Tris-Cl pH 7.5, 5 mM magnesium acetate, 70 mM NH_4Cl and algal hydrolysate. Labelling was carried out for 20 min at 37°. The mixtures were cooled rapidly and then 20 unlabelled amino acids (each at 200 times their concentration in the algal hydrolysate) were added. The mixtures were subsequently treated as described under Experimental Procedures, under incorporation of amino acids, except that treatment by trichloroacetic acid at 90° was omitted

tRNA	ATP	Amino acid bound to tRNA	
μg	mM	pmoles	
40	5	361	360
—	5	11	12
—	—	7	8

tion X must have a high molecular weight. Therefore the crude Fraction X and the active fraction obtained after elution from Sephadex G-200 were examined by centrifugation on linear sucrose gradients. As can be seen in Fig. 4 A, the active component sedimented faster than most of the proteins in the crude Fraction X. Parallel sedimentation of pure ribosomal RNA revealed that the activity was concentrated in a fraction which sedimented slightly slower that 28 S. The sedimentation behaviour of the active component did not change significantly after

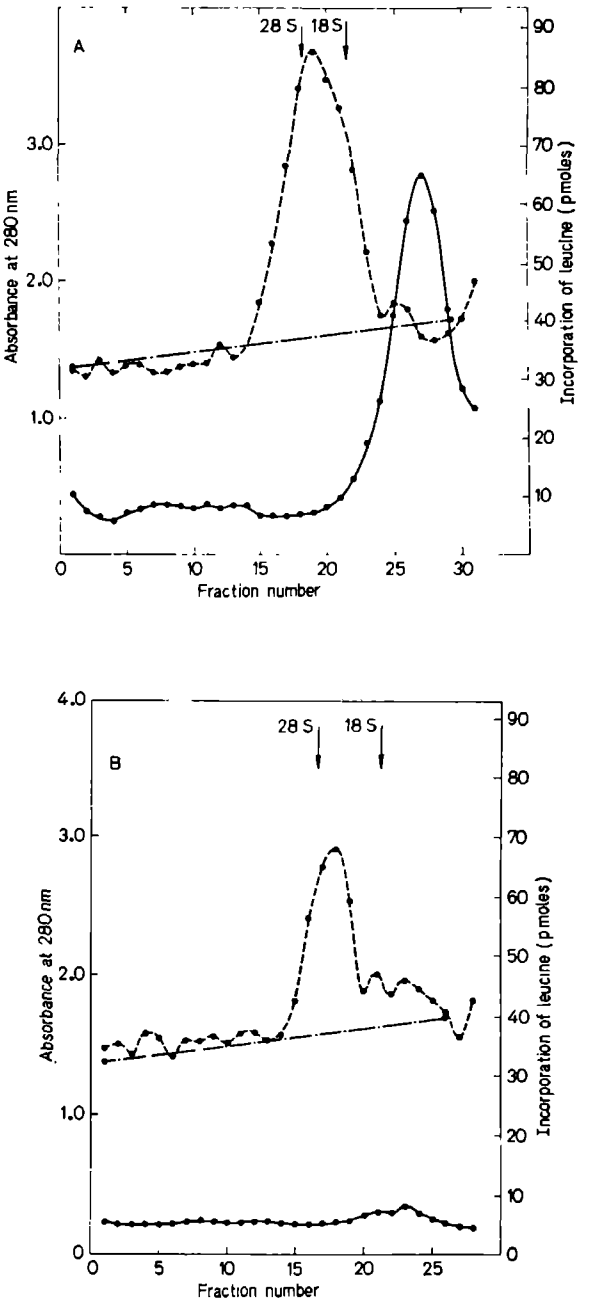


Fig. 4. *Sedimentation in a 10—35 % linear sucrose gradient.* (A) Crude Fraction X, 10.8 mg of protein. (B) Fraction X purified after gel filtration, 1.32 mg of protein. 2 ml containing 5 % sucrose were layered on top of the gradient. Fractions of 15 drops were collected after 12 h centrifugation at 25000 rev./min. For amino acid incorporation, 150 μl of each fraction were assayed as described in Fig. 3. The arrows indicate the position of RNA isolated from rat liver polyribosomes and centrifuged 12 h at 25000 rev./min in a 10 to 35 % linear sucrose gradient containing 0.01 M sodium acetate (pH 5.1) and 0.1 M NaCl. ●——●, Absorbance at 280 nm; ●----●, incorporation of [^{14}C]leucine in the endogenous system; -·-·-·-, control incorporations of [^{14}C]leucine in the presence of 150 μl of 10 or 35 % sucrose

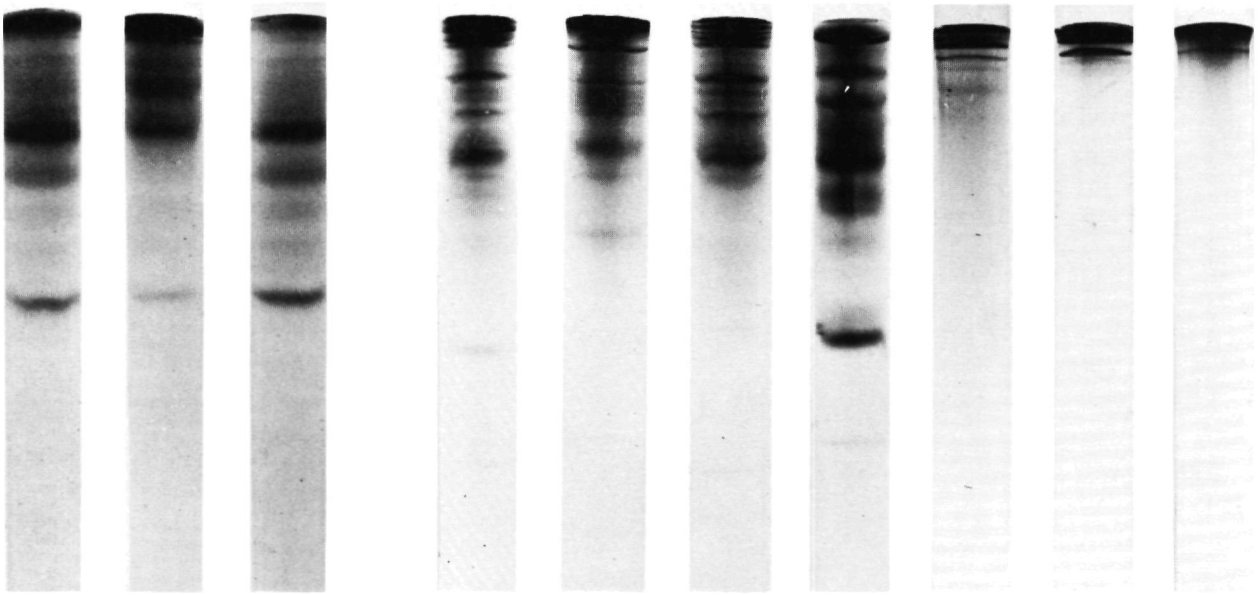


Fig. 5 Fig. 6

Fig. 5. *Polyacrylamide gel electrophoresis.* (A) Total cell sap; (B) Fraction X; (C) Supernatant X

Fig. 6. *Polyacrylamide gel electrophoresis of Fraction X after successive purification steps.* (A) Crude Fraction X; (B) fraction precipitated at 30 % saturation of ammonium sulphate; (C) fraction precipitated between 30 and 60 % saturation of ammonium sulphate; (D) fraction precipitated between 60 and 90 % saturation of ammonium sulphate; (E) C after gel filtration on Sephadex G-200; (F) active fraction after centrifugation in a sucrose gradient; (G) transferase I purified according to Schneir and Moldave [9]

precipitation with ammonium sulphate followed by gel filtration on Sephadex G-200 and concentration on Sephadex G-25 (coarse, Fig. 4 B). The sedimentation profile did not reveal the presence of ribosomal subunits.

The active region of Fraction X purified on a sucrose gradient was submitted to centrifugation in an analytical ultracentrifuge equipped with ultraviolet optics and an automatic scanner. Only one peak was observed which was characterized by a sedimentation coefficient $s_{20} = 26.8$ S.

Polyacrylamide Gel Electrophoresis

Each purification step was controlled by polyacrylamide gel electrophoresis. The starting material revealed a very complex pattern. In the crude Fraction X the slowly moving bands appeared to be predominant (Fig. 5 B). Fig. 6 shows the pattern of the fractions obtained after ammonium sulphate fractionation. As we have mentioned above, all fractions stimulated the incorporation of amino acids into protein. This observation, combined with the protein distribution on the gels after electrophoresis, strongly suggested that the activity was located in the slowly moving region. Further support for this assumption can be derived from the gel pattern of the active fraction eluted from the G-200 column (Fig. 6 E). However, more than five bands were still present after this purification step. A similar result was obtained when the crude Fraction X was subjected to centrifugation in sucrose gradients and the active region isolated (Fig. 6 F).

Hoagland [1] has already considered the possibility that RNA may play a role in the effect of Fraction X. We investigated whether RNA could be demonstrated on the polyacrylamide gel columns. Staining with toluidine blue revealed that this was the case (Fig. 7 B, 7 C). RNA coïncided with the most slowly migrating protein bands. This may mean that the RNA is present as ribonucleoprotein.

We succeeded in isolating RNA from the G-200 fraction. As shown in Fig. 8 A, in crude Fraction X the main bands coïncided with the 18 S and 4 S component. After purification on Sephadex G-200 the 18 S component was concentrated, whereas the concentration of the 4 S component decreased. In addition, a faint zone corresponding with 8 S RNA was present.

4 S RNA could be removed from crude Fraction X by chromatography on DEAE-Sephadex A-50 (Fig. 8 D), whereas 18 S and 8 S RNA could not be detected in the protein-containing fractions after chromatography on DEAE-Sephadex.

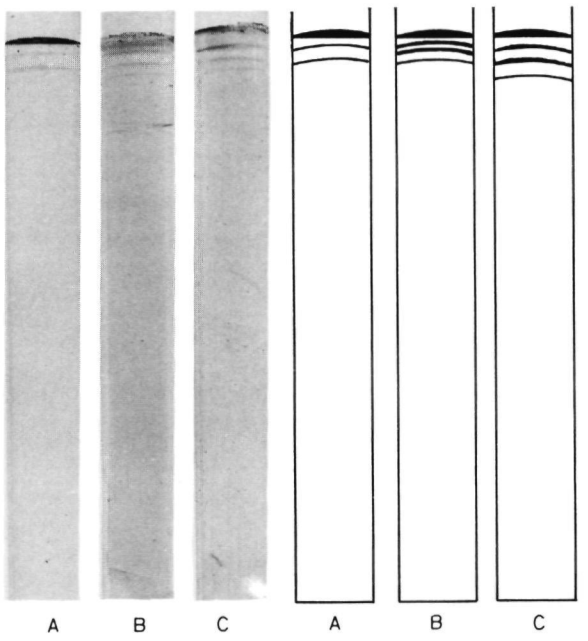


Fig. 7

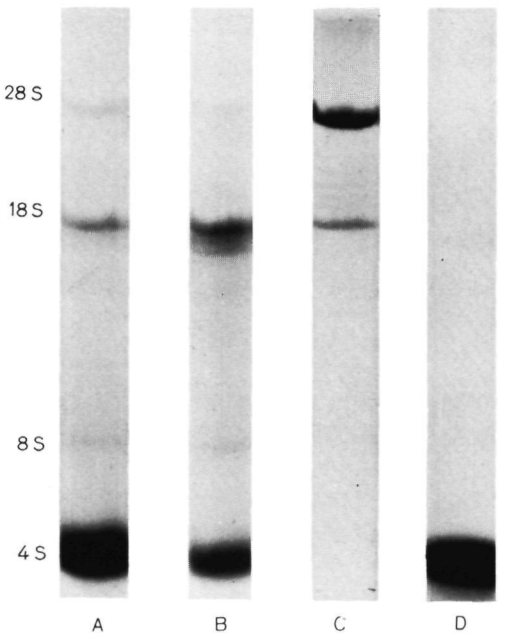


Fig. 8

Fig. 7. *Polyacrylamide gel electrophoresis of the partially purified Fraction X.* Active fraction after centrifugation in a sucrose gradient (A and B); active fraction in eluate from filtration on Sephadex G-200 (C). Staining with toluidine blue O (B and C) and with Sudan Black B or oil red O (A)

Fig. 8. *Polyacrylamide gel electrophoresis of RNA isolated by phenol-dodecyl sulphate extraction from the crude Fraction X (A) and from the purified Fraction X, or $X_{G\text{-}200}$ (B).* (C) shows the pattern of RNA isolated from rat liver polysomes and (D) shows the pattern of the RNA of crude Fraction X eluted from a column of DEAE-Sephadex A-50 by a linear salt gradient (0.5 M NaCl fraction)

Gel electrophoresis also revealed the presence of a small amount of lipoprotein in purified Fraction X (Fig. 7C). This figure illustrates the pattern after prestaining with Sudan Black B. The same results were obtained by staining with oil red O. The lipoprotein presumably originated from membrane fragments which were also observed in electron micrographs of purified Fraction X [23a]. We have shown that they did not contribute to the activity, as deoxycholate treatment did not cause a decrease in stimulatory capacity or change the sedimentation velocity of the active component.

Despite all data hitherto published [1—4, 23] the exact function of Fraction X is still unknown. Our results with the active fraction obtained after chromatography on DEAE-Sephadex A-50 and with RNA prepared from crude or purified Fraction X do not support the idea that RNA is responsible for the activity of Fraction X. This is consistent with the results obtained by Partier and Hultin [4] and Mizrahi [3] who found that stimulation by Fraction X could neither be attributed to tRNA nor to mRNA. Examination of the gel electrophoresis pattern of purified Fraction X demonstrated that it still contained about 5 protein components, some of which occurred as ribonucleoproteins or as lipoproteins. We do not know whether the activity is located in one of these bands or whether more components together stimulate amino acid incorporation. At any rate, the present experiments rule out the possibility that stimulation was caused by the inhibition of ribonuclease activity. Moreover, the presence of transfer factors in Fraction X was not responsible for the latter's activity, since addition of these factors failed to give stimulation. Furthermore, the system depleted from aminoacyl-tRNA-synthetase activity, but saturated with the transfer factors, did not show any stimulation by addition of Fraction X. On the other hand, under these conditions the synthetase-dependent system could be stimulated. Preliminary experiments revealed that in a sucrose gradient the leucyl-tRNA-synthetase activity overlapped the stimulatory region of the purified Fraction X completely. Although aggregation of subunits into active dimeric or tetrameric forms appears to be a common feature for all synthetase [24], the purified enzymes from bacteria [24—34], yeast [35] and bovine pancreas [36] show a sedimentation value ranging

from 1.6 S [36] to 8.6 S [24,32] depending on the number of subunits. However, Bont *et al* [37] found that in crude cell sap from rat liver the synthetase activity was eluted directly after the void volume from a Sephadex G-200 column. This may be explained by aggregation of several amino acid-activating enzymes into a rather stable complex. Such a complex presumably accounts for the effect of Fraction X on the amino acid incorporation described here.

The authors are obliged to Miss Dr. A. Gribnau for assaying ribonuclease inhibitor activity, to Miss J. van Westreenen for technical assistance and to Dr. M. Sluyser for correcting the manuscript. The present investigations have partly been carried out under auspices of the Netherlands Foundation for Chemical Research (S. O. N.) and with financial aid from the Netherlands Organization for the advancement of Pure Research (Z. W. O.).

REFERENCES

1. Hoagland, M., *Cold Spring Harbor Symp. Quant. Biol.* 26 (1961) 153.
2. Hoagland, M. B., and Askonas, B., *Proc. Nat. Acad. Sci U. S. A.* 49 (1963) 130.
3. Mizrahi, I. J., *Biochim. Biophys. Acta*, 108 (1965) 419.
4. Parthier, B., and Hultin, T., *Biochim Biophys. Acta*, 149 (1967) 562
5. Bont, W. S., Rezelman, G., and Bloemendal, H., *Biochem. J.* 95 (1965) 15c.
6. Gribnau, A. A. M., Schoenmakers, J. G. G., and Bloemendal, H., *Arch. Biochem. Biophys.* 130 (1969) 48.
7. Bloemendal, H., Bont, W. S., de Vries, M., and Benedetti, E. L., *Biochem. J.* 103 (1967) 177
8. Bont, W. S., Rezelman, G., Meisner, I., and Bloemendal, H., *Arch. Biochem. Biophys.* 119 (1967) 36.
9. Schneir, M., and Moldave, K., *Biochim. Biophys. Acta*, 166 (1968) 58.
10. Fessenden, J., and Moldave, K., *Biochem. Biophys. Res. Commun.* 6 (1961) 232
11. Skogerson, L., and Moldave, K., *Biochem. Biophys. Res. Commun.* 27 (1967) 568.
12. Sutter, R. P., and Moldave, K., *J. Biol. Chem.* 241 (1966) 1698.
13. Brunngraber, E. F., *Biochem. Biophys. Res Commun.* 8 (1962) 1.
14. Lapidot, Y., de Groot, N., Weiss, M., Peled, R., and Wolman, Y., *Biochim. Biophys. Acta*, 138 (1967) 241.
15. Zamecnik, P. C., Stephenson, M. L., and Scott, J. F., *Proc. Nat. Acad. Sci., U. S. A.* 46 (1960) 811.
16. Konings, R. N. H., and Bloemendal, H., *Eur. J. Biochem.* 7 (1969) 165.
17. Spencer, D., and Whitefeld, P. R., *Arch. Biochem. Biophys.* 117 (1966) 337.
18. Bloemendal, H., *Zone Electrophoresis in Blocks and Columns*, Elsevier, Amsterdam 1963.
19. Loening, U. E., *Biochem. J.* 102 (1967) 251.
20. McDonald, H. J., and Ribeiro, L. P., *Clin. Chim. Acta*, 4 (1959) 458.
21. Pert, J. H., and Kutt, H., *Proc. Soc. Exptl. Biol. Med.* 99 (1958) 181.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* 193 (1951) 265.
23. Beard, N. S., and Armentrout, S. A., *Proc. Nat. Acad. Sci., U. S. A.* 58 (1967) 750.
23a. Benedetti, E. L., unpublished result.
24. Kosakowski, M. H. J. E., and Bock, A., *Eur. J. Biochem.* 12 (1970) 67.
25. Baldwin, A. N., and Berg, P., *J. Biol. Chem.* 241 (1966) 831.
26. Calendar, R., and Berg, P., *Biochemistry*, 5 (1966) 1681.
27. Stulberg, M. P., *J. Biol. Chem.* 242 (1967) 1060.
28. Bruton, C. J., and Hartley, B. S., *Biochem. J.* 108 (1968) 281.
29. Lemoine, F., Waller, J.-P., and van Rapenbusch, R., *Eur. J. Biochem.* 4 (1968) 213.
30. Yaniv, M., and Gros, F., *J. Mol. Biol.* 44 (1969) 1.
31. Yaniv, M., and Gros, F., *J. Mol. Biol.* 44 (1969) 17.
32. Bock, A., *Arch. Microbiol.* 68 (1969) 165.
33. Lemaire, G., van Rapenbusch, R., Gros, G., and Labousse, B., *Eur. J. Biochem.* 10 (1969) 336.
34. Marshall, R. D., and Zamecnik, P. C., *Biochim. Biophys. Acta*, 181 (1969) 454.
35. Makman, M. H., and Cantoni, G. L., *Biochemistry*, 4 (1965) 1434.
36. Preddi, E. C., *J. Biol. Chem.* 244 (1969) 3958.
37. Bont, W. S., and Rezelman, G., *Int. J. Protein Res.* 1 (1969) 193.

C. Vennegoor and H. Bloemendal
Laboratorium voor Biochemie, Universiteit van Nijmegen
Geert Groteplein Noord 21, Nijmegen, The Netherlands

Eur. J. Biochem. 26 (1972) 462–473

Occurrence and Particle Character of Aminoacyl-tRNA Synthetases in the Post-Microsomal Fraction from Rat Liver

Claudia VENNEGOOR and Hans BLOEMENDAL

Laboratorium voor Biochemie, Universiteit van Nijmegen

(Received October 5/November 22, 1971)

A post-microsomal fraction (Fraction X) was isolated from cell sap from rat liver by centrifugation at 105000 × *g* for 15 h. The activity of a number of aminoacyl-tRNA synthetases was considerably higher in the resulting pellet than in the supernatant. These enzymes (glutaminyl-, isoleucyl-, leucyl-, lysyl-, and methionyl-tRNA synthetase) were purified by gel filtration on Sephadex G-200, chromatography on DEAE-Sephadex A-50 and on hydroxyapatite. As compared with crude cell sap a 120- to 170-fold purification was achieved.

In all purification steps, including additional centrifugation on sucrose gradients or isoelectric focusing, the major peak of activity of the five enzymes coincided.

In the electron microscope circular and rectangular particles, 11–14 nm in size, could be visualized. From these findings we suggest that in rat liver cells, if not in all animal cells, part of the aminoacyl-tRNA synthetases occur in a particulate state.

A post-microsomal fraction, called Fraction X, was first described by Hoagland in 1961 [1]. Different functions have been ascribed to this fraction, for instance messenger RNA [2], interaction between ribosomes and messenger RNA [3,4] and transfer activity [5]. Recently we have reported that amino-acid-activating enzymes might play a major role in the observed stimulation of incorporation of amino acids [6].

In connection with this we investigated the distribution of aminoacyl-tRNA synthetases (amino acid-RNA ligases) in this fraction in more detail.

The synthetases have further been purified by chromatography on DEAE-Sephadex A-50 and hydroxyapatite. They have been characterized by electrophoresis on polyacrylamide gels, centrifugation in sucrose gradients, isoelectric focusing and in the electron microscope.

MATERIALS AND METHODS

Materials

^{14}C-Labeled amino acids, [^{35}S]methionine, and tetra-sodium [^{32}P]pyrophosphate were purchased from the Radiochemical Centre (Amersham, England). GTP, pyruvate kinase and bovine serum albumin, were supplied by Boehringer Mannheim GmbH (Mannheim, Germany).

Phospho*enol*pyruvate (sodium salt), ATP and L-amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Dithiothreitol (Clelands' reagent) was obtained from Calbiochem (Los Angeles, California). Inorganic pyrophosphatase(from bakers' yeast) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Tetrasodium pyrophosphate, ammonium sulphate and glycerol were purchased from Merck AG (Darmstadt, Germany). Polyethylene glycol (Carbowax 6000) was obtained from Fluka AG. Chemische Fabrik (Buchs, Switzerland). Sephadex G-25 (coarse), Sephadex G-200 and DEAE-Sephadex A-50 were products from Pharmacia (Uppsala, Sweden).

Bovine α and β-crystallin were gifts of Dr G. van Kamp. Hydroxyapatite was prepared according to Levin [7].

Buffer Solutions

Medium A consisted of 50 mM Tris-Cl pH 7.6, 25 mM KCl and 5 mM magnesium acetate. Phosphate buffers were, unless stated otherwise, composed of potassium phosphate and contained 10% (w/v) glycerol. To the buffer solutions either dithiothreitol (1 mM) or 2-mercaptoethanol (5 mM) were added immediately before use.

Enzymes. Aminoacyl-tRNA synthetases (EC 6.1.1); inorganic pyrophosphatase (EC 3.6.1.1).

Preparation of Subcellular Fractions from Rat Liver

Total cell sap, supernatant X and fraction X were prepared as described earlier [6]. When the isolation was performed on a large scale, the microsomes were sedimented by centrifugation at $75000 \times g$ for 150 min in rotor 30 of the Spinco model-L preparative ultracentrifuge.

Total cell sap, supernatant X and fraction X were freed from endogenous amino acids and ATP by gel filtration on Sephadex G-25 (coarse), equilibrated in medium A. The protein-containing fractions were concentrated by addition of 1 mg dry Sephadex G-25 (coarse) per 5 ml protein solution; after 30 min at 2 °C the Sephadex was removed by centrifugation at 3000 rev./min for 5 min in a Martin Christ junior centrifuge. Medium A, containing 2 M sucrose was added to a final concentration of 0.35 M. The fractions were stored at — 20 °C in small portions containing 10—17 mg protein/ml.

In a number of experiments supernatant X was freed from pH 5 enzymes. This fraction, devoid of glutaminyl-, isoleucyl, leucyl-, lysyl-, and methionyl-tRNA synthetase, was added to the incubation mixture when formation of aminoacyl-tRNA was assayed.

The solution was adjusted to pH 5.2 by gradual addition of 1 N acetic acid. The resulting suspension was stirred for 1 h in an ice bath and centrifuged at 4500 rev./min in the Martin Christ Jr. centrifuge. The supernatant was adjusted to pH 7.6 by addition of 1 N KOH. Passage through Sephadex G-25 (coarse) and storage of the protein was as described above.

Polyribosomes were isolated according to the method described by Bloemendal *et al.* [8].

Isolation of RNA from Rat Liver

Transfer RNA was isolated from rat liver essentially according to the method of Brunngraber [9], with modifications as described by Konings [10]. The tRNA was stripped by incubation for 90 min at 37 °C in 1.8 M Tris-Cl pH 8.0.

Ribosomal RNA was isolated as described earlier [6].

Assay for Amino-Acid-Dependent ATP—Pyrophosphate Exchange

The activation of amino acids was measured by the amino-acid-dependent ATP—pyrophosphate exchange assay [11]. The reaction mixture (0.25 ml) contained 100 mM Tris-Cl pH 7.6, 7 mM $MgCl_2$, 5 mM ATP (neutralized with KOH), 2 mM L-amino acid, 5 mM KF, 2 mM tetrasodium pyrophosphate (20000—100000 counts/min per assay), 2 mM dithiothreitol, 250 μg bovine serum albumin and limiting amounts of enzyme (dissolved in phosphate buffer). In blanks amino acid was omitted. After incubation at 37 °C for 10 min the reaction was stopped and ATP adsorbed to Norit A as described by Lemoine *et al.* [12], except that the volumes were twice as large. The suspension was filtered through glass-fibre paper (Whatman GF/A) and the filter was washed three times with 10-ml portions of distilled water. The paper was glued onto an aluminium planchet with the Norit surface down [13], dried and counted in a thin-window gas-flow counter.

One unit of enzyme activity is defined as that amount incorporating 1 μmol [^{32}P]pyrophosphate into ATP in 10 min at 37 °C.

Assay for Aminoacyl-tRNA Formation

The incubation mixture (0.2 ml) contained 50 mM Tris-Cl pH 7.6, 25 mM KCl, 5 mM magnesium acetate, 5 mM ATP (neutralized with KOH), 0.125 mM ^{14}C-labeled L-amino acid (or L-[^{35}S]methionine) (25 μCi), 2 mM dithiothreitol, 200 μg rat liver tRNA 100 μg bovine serum albumin and limiting amounts of enzyme. Enzymes were diluted in medium A, containing 0.35 M sucrose and 2 mg/ml (w/v) albumin. In blanks ATP was omitted. Incubation was at 37 °C for 10 min. The reaction was stopped by addition of 5 ml 5% trichloroacetic acid (w/v). The tubes were kept at 0 °C for 30 min. After centrifugation the precipitate was collected on a Whatman glass-fibre paper. Radioactivity was measured in a liquid scintillation spectrophotometer [6].

Incubation mixtures which contained [^{14}C]cysteine, [^{14}C]serine or [^{14}C]tryptophan showed high blank values. In these mixtures the reaction was stopped essentially as described by Muench [14].

One unit of enzyme is equivalent to the formation of 1 nmol of aminoacyl-tRNA in 10 min at 37 °C.

Assay for Incorporation of Amino Acids

Incorporation of [^{14}C]leucine in the endogenous system was measured as described earlier [6].

Assay of Inorganic Pyrophosphatase

Inorganic pyrophosphatase was assayed according to the method described by Heppel [15]. As control for activity inorganic pyrophosphatase from yeast was used.

Protein Determination

Protein was measured by the method of Lowry *et al.* [16] using bovine serum albumin as standard. The standards and blanks contained the same concentration of 2-mercaptoethanol or dithiothreitol as did the protein samples. As 2-mercaptoethanol and dithiothreitol interfere strongly in this assay [17,18], all samples were diluted ten-fold.

When the protein concentration in the column effluent was too small to enable a reliable determination, the protein content was estimated after dialysis by measuring the absorbance at 280 and 260 nm according to Warburg and Christian [19].

Purification of Aminoacyl-tRNA Synthetase from Fraction X

Precipitation with Ammonium Sulphate. Fraction X (400—450 mg protein) was concentrated by precipitation of protein between 30 and 60% saturation with ammonium sulphate [6]. After centrifugation the precipitate was dissolved in 3 ml of medium A and dialyzed during 3 h against this medium. A slight precipitate, which appeared during the course of the dialysis procedure, was removed by centrifugation.

Gel Filtration on Sephadex G-200. The sample (7—8 ml) was applied immediately to a column of Sephadex G-200, equilibrated in Medium A. In order to keep the filtration step short a column (column size 58×4 cm, LKB) was used with a peristaltic pump (velocity 15 ml/h) by which the sample and the buffer were introduced into the bottom of the column and pumped upward. This procedure improved the separation of the active fraction (which was the first protein peak [6]) from the inactive protein.

When no further purification was performed, the active fraction was concentrated by addition of Sephadex G-25 (coarse) and subsequent centrifugation. The solution was made 0.35 M in sucrose, frozen in small portions and stored at −20 °C.

When purification was continued, glycerol was added to a final concentration of 10% (w/v) and the protein was dialyzed during at least 3 h against 1 l of 20 mM phosphate buffer pH 7.5. The buffer was changed twice.

Chromatography on DEAE-Sephadex A-50. After dialysis, the sample was applied onto a column of DEAE-Sephadex A-50 (column size 12×1.2 cm), equilibrated in 20 mM phosphate buffer pH 7.5. The column was washed with 50 ml buffer and the enzymes were eluted with a linear salt gradient formed of 75 ml 0.02 M phosphate buffer pH 7.5 and 75 ml 0.5 M phosphate buffer pH 6.5. The flow rate was 15 ml/h and fractions of approximately 2 ml were collected. Fractions which were 0.09 to 0.2 M in phosphate were pooled, concentrated by dialysis against 1 l of 20 mM potassium phosphate buffer pH 7.0, containing 20% (w/v) polyethylene glycol, 10% (w/v) glycerol and 1 mM dithiothreitol, and subsequently dialyzed against the same buffer without polyethylene glycol.

Chromatography on Hydroxyapatite. The diffusate was loaded on a column of hydroxyapatite (column size 10×1.2 cm), equilibrated in 20 mM phosphate buffer pH 7.0. The column was washed with 20 ml buffer. A linear gradient was applied ranging from 0.02 to 0.7 M phosphate pH 7.0. The total volume of the effluent was 150 ml. The flow rate was adjusted to 9 ml/h with the aid of a peristaltic pump and 2-ml fractions were collected.

The enzyme activities emerged at a phosphate concentration of about 0.35 M.

Centrifugation on Sucrose Gradients

Centrifugation on linear 10—35% sucrose gradients was performed as described earlier [6].

Exponential isokinetic sucrose gradients were prepared and analyzed essentially according to the method of McCarty *et al.* [20]. Isokinetic gradients were prepared in centrifuge tubes of the IEC SB-283 rotor. The sucrose solutions were in a buffer containing 20 mM potassium phosphate pH 7.5, 0.1 M KCl and 1 mM dithiothreitol. A particle density of 1.3 was assumed. The concentrations of sucrose solutions were, according to data of Noll [21], 15% (w/v) in the mixing vessel and 32.8% in the reservoir. Six identical gradients were generated simultaneously. The samples (0.5 ml) which had been dialyzed against the buffer in which the sucrose solutions were prepared, were carefully layered on top of the gradient. Centrifugation at 40000 rev./min and 2 °C was carried out in the IEC ultracentrifuge. After the run, fractions of approximately 0.27 ml were collected by top unloading of the gradient with the aid of a 2 M sucrose solution.

Isoelectric Focusing

Isoelectric focusing of purified synthetases was performed essentially as described by Surguchev *et al.* [22] with the following modifications. Ampholines pH 5—8 were used. Both dense and light solution contained 1 mM dithiothreitol. 1.5—2 mg of protein in 7—10 ml of 20 mM potassium phosphate buffer pH 7.5 were mixed with the dense solution. The cathode solution, containing 0.4 ml triethanolamine, 12 g sucrose and 14 ml distilled water, was poured into the lower part of the column. The anode solution, 0.1 ml phosphoric acid and 10 ml distilled water, was layered 1.5 cm above the upper electrode. The column was thermostatted at 0—2 °C. Maximally 1 W was applied onto the column. The duration of the run was 60—72 h at a final current of 1.2 mA and 800 V.

Electrophoresis on Polyacrylamide Gels

Separation of proteins on polyacrylamide gels was performed as described by Bloemendal [23] except that the composition of the gel was 4.5% acrylamide, 0.24% bisacrylamide 0.085 M Tris-borate pH 8.9, 0.4% 3-dimethylaminopropionitrile,

0.003% $K_3Fe(CN)_6$ and 0.125% ammonium persulphate. All solutions were degassed before they were poured into the tubes.

Electrophoresis in sodium dodecylsulphate [24] was carried out as described by Weber and Osborn [25].

Electron Microscopy

Enzyme preparations were dialyzed overnight against 0.1 M ammonium acetate buffer pH 7.0 at 2 °C. Droplets of fluid were placed on a grid covered with a carbon film and almost completely removed with a piece of filter paper. A small drop of either 2% potassium phosphotungstate pH 6.7 or 0.5% uranyl oxalate pH 7.0 [26] or uranyl formate [27] was applied to the grid. Excess of negative-stain solution was removed with filter paper. Electron micrographs were taken with a Philips EM-300 microscope, operated at 60 kV, fitted with a cooling device.

RESULTS AND DISCUSSION

Aminoacyl-tRNA Synthetase Activity

Fraction X stimulates the incorporation of amino acids in the presence of polyribosomes [1–6]. This fraction showed considerable aminoacyl-tRNA synthetase activity [6]. When fraction X was centrifuged in a linear sucrose gradient, amino-acid-activating enzymes appeared to be present in the same region in which activity of fraction X was located. In Fig. 1 ist is shown that leucyl-tRNA synthetase activity was concentrated in two different regions of the gradient. The corresponding sedimentation coefficients were 25 S and 18 S, respectively. Less than 20% of the activity of leucyl-tRNA synthetase remained in supernatant X. When the distribution of 20 aminoacyl-tRNA synthetases in total cell sap, supernatant X and fraction X was determined, it appeared that the amino-acid-activating enzymes were not uniformly distributed throughout these fractions (Table 1). In order to enable comparison of the distribution of the different synthetases, the total number of units in the total cell sap had been equalized to 100% for each enzyme. (Enzyme activity is expressed in units, defined as specific activity times mg protein.) The recovery of the individual enzyme activities was markedly different. In the case of lysyl-tRNA synthetase about 40% was lost. Presumably this reflects a different degree of stability of the individual enzymes, a phenomenon which has been reported for the synthetases from *Escherichia coli* [28]. Loss of activity however, was not paralleled with a loss of protein. The fractions derived from total cell sap contained together as much protein as the starting material.

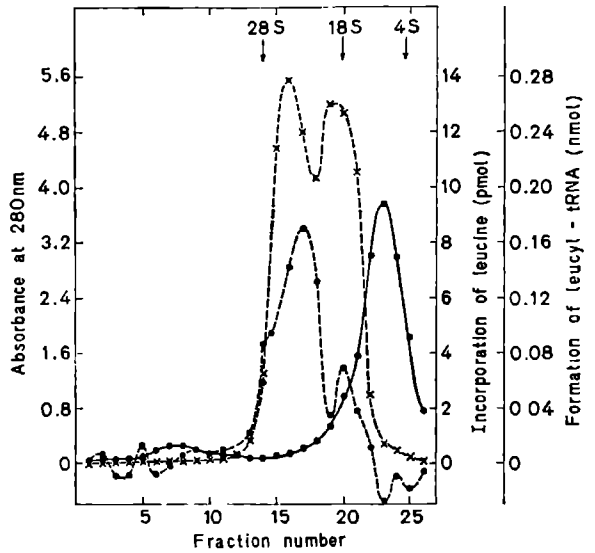


Fig. 1. *Sedimentation of fraction X in a 10 to 35% linear sucrose gradient.* An aliquot of 20 mg protein was layered on top of the gradient. Fractions of 13 drops were collected after 12 h of centrifugation at 25000 rev./min. For incorporation of amino acids, 50 µl of each fraction were assayed. The incubation mixtures (0.25 ml) contained 0.5 mg of supernatant X protein and 100 µg of polyribosomes. For formation of leucyl-tRNA 25 µl of each fraction was assayed. The incubation mixtures (0.2 ml) were as described in Experimental Procedures. The arrows indicate the position of tRNA and of RNA isolated from rat liver polyribosomes after 12 h of centrifugation at 25000 rev./min in a 10 to 35% linear sucrose gradient containing 0.01 M sodium acetate (pH 5.1) and 0.1 M NaCl. ●----●, stimulation of incorporation of [^{14}C]leucine; ×----×, formation of [^{14}C]leucyl-tRNA; ●——●, absorbance at 280 nm

We also compared the specific activity of aminoacyl-tRNA synthetases in fraction X with their specific activity after purification of this fraction by gel filtration on Sephadex G-200. A number of synthetases accumulated in the first protein peak. This fraction was completeley devoid of glycyl-, histidyl-, seryl-, and tryptophanyl-tRNA synthetase activity, whereas the specific activity of alanyl-, asparagyl-, cysteinyl-, threonyl- and tyrosyl-tRNA synthetases was lower than in fraction X before purification (Table 2). The behaviour of aspartyl-tRNA synthetase was quite unexpected. While the activity of this enzyme remained predominantly in supernatant X (Table 1), its activity was considerably enhanced in the purified fraction X (Table 2).

Purification of Five Aminoacyl-tRNA Synthetases

As glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase accumulated in fraction X, showing only a very low activity in supernatant X, we attempted to further purify these

Table 1. *Distribution of aminoacyl-tRNA synthetases in total cell sap, supernatant X and fraction X*
Enzyme activity was measured by means of formation of aminoacyl-tRNA as described in Experimental Procedures. The total number of units was calculated from the specific activity of the distinct enzyme (expressed in nmol amino acid attached to tRNA in 10 min at 37 °C per mg protein) times mg protein in each fracion. The values for supernatant X and fraction X are given proportional to the number of units present in total cell sap

Substrate	Total cell sap	Supernatant X	Fraction X	Recovery
	units	%	%	%
Alanine	9630	69	15	84
Arginine	4448	53	47	100
Asparagine	5786	52	27	79
Aspartic acid	5366	76	23	99
Cysteine	3280	74	20	94
Glutamic acid	2021	35	56	91
Glutamine	1010	10	66	76
Glycine	4146	60	20	80
Histidine	1942	66	19	85
Isoleucine	123	12	49	61
Leucine	682	15	61	76
Lysine	6324	5	55	60
Methionine	774	9	57	66
Phenylalanine	1260	60	37	97
Proline	3503	53	31	84
Serine	3503	68	30	98
Threonine	8567	52	27	79
Tyrosine	1010	79	22	101
Tryptophan	879	90	20	110
Valine	682	37	48	85

Table 2. *Specific activity of aminoacyl-tRNA synthetases in fraction X and in purified fraction X*
Specific activity was measured as described in Table 1

Substrate	Specific activity	
	Fraction X	Fraction X after purification on Sephadex G-200
	units/mg protein	
Alanine	7.1	0.08
Arginine	10.3	32.9
Asparagine	7.8	1.0
Aspartic acid	6.2	40.9
Cysteine	3.3	3.0
Glutamic acid	5.6	13.8
Glutamine	3.3	10.5
Glycine	4.1	0
Histidine	1.8	0
Isoleucine	0.3	0.8
Leucine	2.1	8.3
Lysine	17.4	58.9
Methionine	2.2	8 7
Phenylalanine	2.3	4 0
Proline	5.4	5.9
Serine	5.2	0
Threonine	11.6	2.4
Tyrosine	1.1	0.1
Tryptophan	0.9	0
Valine	1.6	4 0

enzymes. We applied chromatography on DEAE-Sephadex A-50 and hydroxyapatite, techniques which had earlier succesfully been used for the purification of bacterial synthetases [12—14, 29—35]. In addition, gel filtration on Sephadex G-200 was performed as a first step. The results of the different purification steps are summarized in Table 3. Enzyme activity has been measured by means of amino-acid-dependent ATP—pyrophosphate exchange.

Activity of glutaminyl-tRNA synthetase could not be measured when no tRNA was present in the reaction mixture. Therefore, tRNA, from rat liver (200 μg) was added when glutamine-dependent ATP—pyrophosphate exchange had to be determined [36—38]. When Tris-maleate-KOH buffer was added to the incubation mixtures, we did not notice a shift in optimal pH to a lower value as reported for rat liver glutamyl-tRNA synthetase [38].

The active protein peak from the Sephadex G-200 step had an $A_{280} : A_{260}$ absorbance ratio of 0.72—0.67 which, according to Warburg and Christian [19], indicates the presence of 10—12% nucleic acid.

The purification which was achieved in the subsequent DEAE-Sephadex step was rather low and was accompanied with a considerable loss of activity. However, as the RNA present in the Sephadex G-200 effluent was removed by this procedure, this step was included. The removal of RNA increased the lability of the enzymes. This phenomenon was also observed by Deutscher for rat liver glutamyl-tRNA synthetase [39]. Therefore, when the enzymes had to be stored after this step, the protein was dialyzed against potassium phosphate buffer pH 7.0 containing 50% (w/v) glycerol in order to stabilize the enzymes [34, 39, 40]. By this procedure the protein was concomitantly concentrated two-fold.

The purification of glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase after the gel filtration step is illustrated in Fig. 2 and Fig. 3. Synthetase activity was localized by measuring the formation of aminoacyl-tRNA (Fig. 2).

In order to enhance formation of isoleucyl-tRNA, supernatant X freed from pH 5 enzymes, had to be added to the assay mixture (Bont, W. S., unpublished results). In the presence of this fraction the formation of glutaminyl- and lysyl-tRNA was also stimulated.

The main peak of synthetase activities emerged from the column between 0.10 and 0.18 M phosphate. The capacity to attach amino acids to tRNA coincided with the amino acid activation as measured by ATP—pyrophosphate exchange.

Fig. 2 also shows the stimulation of incorporation of amino acids. Maximal stimulation coincided with maximal activity of aminoacyl-tRNA synthetases. These data and the results presented in Table 1 and Table 2 strongly suggest that, in contrast to conclusions of others [1—5], amino-acid-activating enzymes

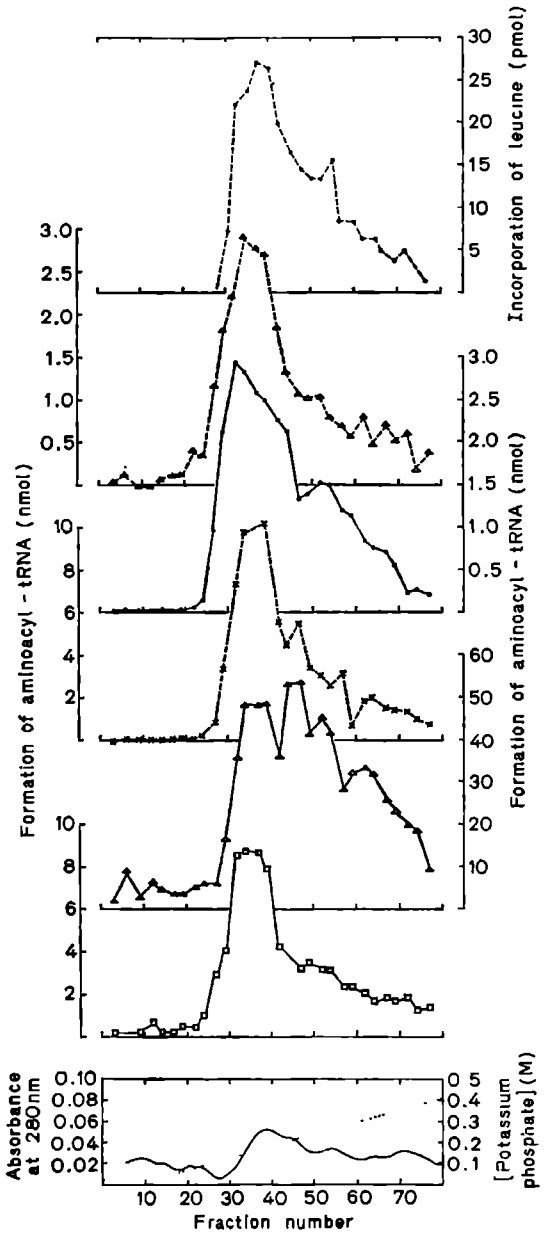


Fig. 2. *Elution from DEAE-Sephadex A-50 of proteins of fraction X.* The first protein peak from the Sephadex G-200 step (9.5 mg protein in 19.5 ml) was dialyzed and applied onto a DEAE-Sephadex column, washed with 0.02 M potassium phosphate buffer pH 7.5 and eluted with a potassium phosphate–pH gradient (100 ml). Fractions were 1.22 ml. For incorporation of amino acids, 50 µl of each fraction was assayed. The incubation mixtures (0.25 ml) contained 0.4 mg of supernatant X protein and 100 µg of polyribosomes. For determination of synthetase activity each fraction was diluted, when necessary, in 100 mM Tris-Cl pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 1 mM dithiothreitol in the proper proportion in order to achieve limiting enzyme concentration in the incubation mixtures. The incubation mixtures were as described in Experimental Procedures, except that the reaction mixture was reduced to 0.1 ml and 100 µg of supernatant X freed from pH 5 enzymes was added instead of albumin. Synthetase activities have been expressed by formation of aminoacyl-tRNA in nmol per column fraction. ●----●, stimulation of incorporation of [^{14}C]leucine; △----△, glutaminyl-tRNA synthetase; O——O, isoleucyl-tRNA synthetase; ×----×, leucyl-tRNA synthetase; △——△, lysyl-tRNA synthetase; □——□, methionyl-tRNA synthetase; ——, absorbance at 280 nm; ·····, concentration of potassium phosphate

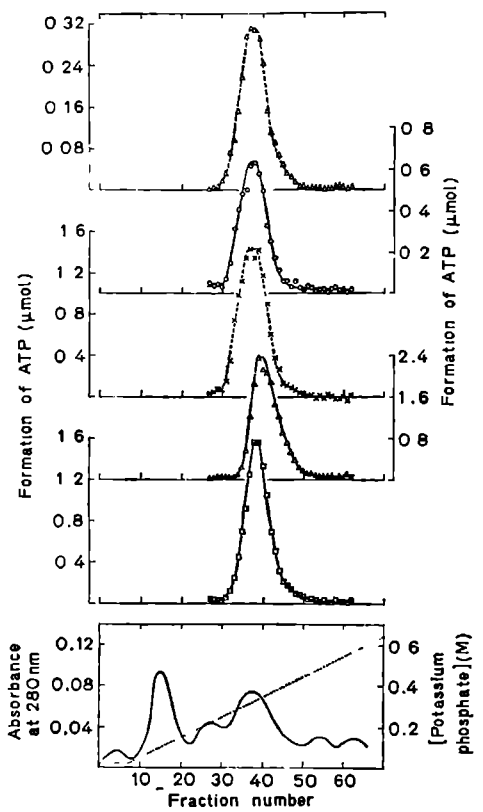


Fig. 3. *Elution from hydroxyapatite of proteins of fraction X.* The active fraction from the DEAE-Sephadex step (5.1 mg protein in 46 ml) was dialyzed and applied onto a column of hydroxyapatite, washed with 20 mM potassium phosphate buffer pH 7.0 and eluted with a linear gradient of potassium phosphate (150 ml). Fractions were 2.0 ml. Synthetase activity was assayed by means of amino-acid-dependent ATP-pyrophosphate exchange. The enzyme activities have been expressed by formation of ATP in µmol per column fraction. △--- △, glutaminyl-tRNA synthetase; O——O, isoleucyl-tRNA synthetase; ×----×, leucyl-tRNA synthetase; △——△, lysyl-tRNA synthetase; □——□, methionyl-tRNA synthetase; ——, absorbance at 280 nm; ·····, concentration of potassium phosphate

Table 3. *Purification of glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase*

Enzyme activity was measured by means of amino-acid-dependent ATP-pyrophosphate exchange. The values for total protein have been calculated for the isolation of synthetases from 50 rat livers

Step	Protein	Specific activity of tRNA synthetase for					Recovery of tRNA synthetase activity for					Purification of tRNA synthetase for				
		Gln	Ile	Leu	Lys	Met	Gln	Ile	Leu	Lys	Met	Gln	Ile	Leu	Lys	Met
	mg	units/mg protein					%	%	%	%	%	-fold				
Total cell sap	14830	0.026	0.106	0.197	0.036	0.081	100	100	100	100	100	1	1	1	1	1
Pellet (fraction X)	2183	0.149	0.336	0.673	0.181	0.348	84	47	50	74	63	5.7	3.4	3.4	5.0	4.3
Sephadex G-200 eluate	114	1.10	3.90	6.89	2.92	4.60	32	28	27	55	44	42	37	35	72	57
DEAE-Sephadex eluate	18	1.72	5.22	11.8	3.88	6.77	8.0	6.0	7.3	9.7	10.2	66	49	60	80	84
Hydroxyapatite[a]	3.3	3.56	13.6	23.6	13.0	11.8	3.0	2.9	2.7	3.7	3.2	137	128	120	168	146

[a] Four preparations were combined for chromatography on hydroxy-apatite.

are responsible for the stimulation of incorporation of amino acids by this fraction.

In Fig. 3 the elution pattern of the five synthetases from the column of hydroxyapatite is shown. An ultraviolet-absorbing peak was eluted at 10 mM potassium phosphate buffer pH 7.0 which did not contain synthetase activity.

Also in the preceding wash of the column of hydroxyapatite no synthetase activity could be measured. The most striking result in this purification step is that again the peaks of activity of glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase coincided.

From the results summarized in Table 3, Fig. 2 and Fig. 3, one has to conclude that the five enzymes resist separation by the purification procedures described.

CHARACTERIZATION OF THE PURIFIED SYNTHETASES

Sedimentation Analysis

The results described above suggested that the five enzymes were aggregated into a rather stable complex. In order to verify this assumption, we also examined the sedimentation behaviour of the purified enzymes in a sucrose gradient. The result is depicted in Fig. 4. It has been reported that amino-acid-activating enzymes have molecular weights of about 100000 to 200000 and sedimentation coefficients of 5 to 8 S [30,32–35,40–47). However, the five enzymes studied revealed a considerably higher sedimentation value. Assuming a particle density of 1.3, a value of 18.2 S can be derived for glutaminyl-, lysyl- and methionyl-tRNA synthetase. Isoleucyl- and leucyl-tRNA synthetase showed each two peaks of activity which were localized in fractions in which proteins were expected with a sedimentation value of 12.5 and 18 S, respectively. Stimulation of incorporation of amino acids coincided with the synthetases which sedimented at 18 S. No synthetase activity or capacity to stimulate incorporation of amino acids could be detected in fractions 35 to 40, in which protein might be expected with a sedimentation coefficient up to 28 S.

When the eluate from the column of hydroxyapatite was centrifuged in an analytical ultracentrifuge, a rather broad peak was observed which was characterized by sedimentation coefficients ranging from $s_{20,w} = 17.9$ to 21.2 S.

The sedimentation behaviour of the synthetases after purification by chromatography on DEAE-Sephadex and hydroxyapatite differed from the sedimentation behaviour of less purified enzymes. The less purified amino-acid-activating enzymes showed, in addition to 18-S components, components with a sedimentation value of approximately 25 S (compare Fig. 1). Stimulatory activity for incorpora-

tion of amino acids was also located in fractions corresponding to higher sedimentation coefficients. However, the purified synthetases sedimented only at 18 S. Presumably the amino-acid-activating enzymes had been converted to material which is characterized by the latter sedimentation coefficient.

Isoelectric Focusing

In a final attempt to separate the amino-acid-activating enzymes, we subjected the proteins present in the eluate from the column of hydroxyapatite to isoelectric focusing. Two peaks of ultraviolet-absorbing material were found, with *pI* 7.0 and 5.7, respectively. All five enzymes showed a peak of activity in fractions with *pI* 5.7. Smaller peaks of activity were observed at pH 6.1 and/or 6.5 (Fig. 5) *cf.* [48]. Activity was measured both by formation of aminoacyl-tRNA and by amino-acid-dependent ATP–pyrophosphate exchange. The curves of activity coincided in both methods. This is in contrast with the results of Surguchev *et al.* [22]. The capacity to stimulate incorporation of amino acids was also examined in the fractions which were obtained after isoelectric focusing. Stimulation coincided with the fractions which had been focused at pH 5.7. In the other fractions no stimulation was detected.

Electrophoresis on Polyacrylamide Gels

In a 4.5% polyacrylamide gel six bands were visible from the eluate of the column of Sephadex G-200 (Fig. 6A). The protein isolated after chromatography on DEAE-Sephadex revealed a sharp and a diffuse band (Fig. 6B). Likewise the band pattern of proteins obtained after chromatography on hydroxyapatite contained one sharp and a diffuse band (Fig. 6C). However, the sharp protein band hardly penetrated into the gel. This band may represent protein which had been aggregated during the electrophoretic procedure. The diffuse band had a mobility comparable with a (diffuse) band which was also observed after electrophoresis of the protein from the Sephadex G-200 eluate. Probably this band represents the active material in fraction X.

Sodium dodecylsulphate dissociates the protein complex into eight components (Fig. 6D). Molecular weights of the proteins were estimated according to the procedure of Weber and Osborn [25] (*cf.* Fig. 6D). The same pattern was observed when protein from the peak which had been focused at pH 5.7 was sub-

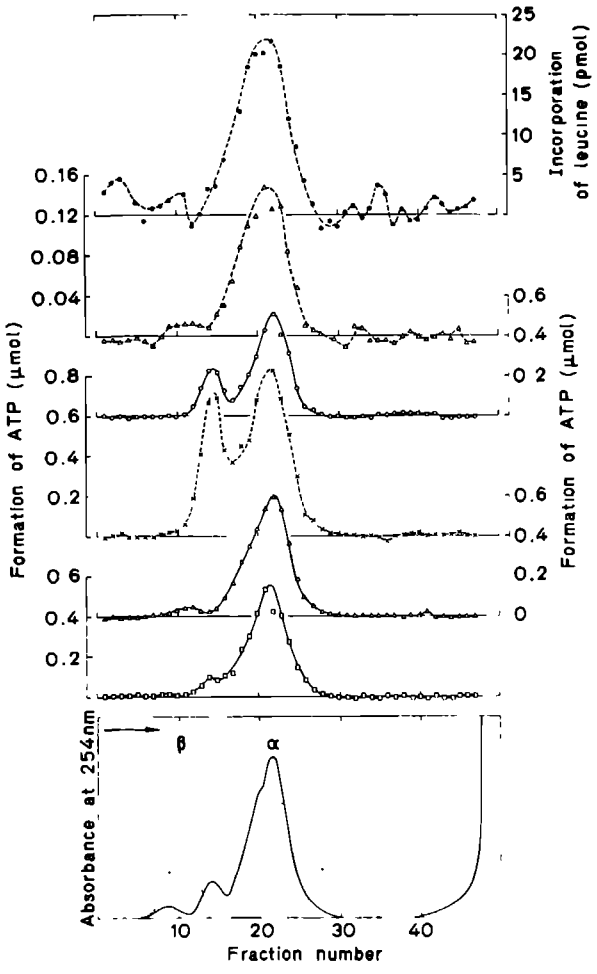


Fig. 4. *Sedimentation in an isokinetic sucrose gradient.* Fraction X was purified by gel filtration on Sephadex G-200 and by chromatography on DEAE-Sephadex A-50 and hydroxyapatite. The active fraction was concentrated by dialysis *in vacuo* against 20 mM potassium phosphate buffer pH 7.5, containing 0.1 M KCl and 1 mM dithiothreitol. 0.324 mg protein in 0.5 ml were layered on top of an isokinetic gradient. α-Crystallin (sedimentation coefficient 19.8 S) and β-crystallin (sedimentation coefficient 7.9 S) were dissolved in the buffer as used for dialysis of purified fraction X. 0.406 mg α-crystallin and 0.080 mg β-crystallin, each in 0.5 ml, were layered on separate, identical gradients. Fractions of 0.266 ml were collected, after 16 h of centrifugation at 40000 rev./min, by top unloading of the gradient with a 2 M sucrose solution. The absorbance was measured continuously at 254 nm. The bottom of the gradient is marked by the rapid increase in absorbance due to the 2 M sucrose solution. Each fraction was diluted fourfold in 1 mM potassium phosphate buffer pH 7.5, containing 1 mg/ml (w/v) albumin, 10% (w/v) glycerol and 1 mM dithiothreitol. For incorporation of amino acids 75 µl of each diluted fraction was assayed. The incubation mixtures (0.25 ml) contained 0.5 mg supernatant X protein and 100 µg polyribosomes. Synthetase activity was measured as described in Fig. 4. The enzyme activities have been expressed by formation of ATP in µmol per gradient fraction. The arrow indicates the direction of sedimentation. ●----●, stimulation of incorporation of [^{14}C]leucine; △----△, glutaminyl-tRNA synthetase; ○——○, isoleucyl-tRNA synthetase; ×- - -×, leucyl-tRNA synthetase; ▲——▲, lysyl-tRNA synthetase; □——□, methionyl-tRNA synthetase; - —, absorbance at 254 nm of fraction X in relative units; ·····, absorbance at 254 nm of α- and β-crystallin in relative units

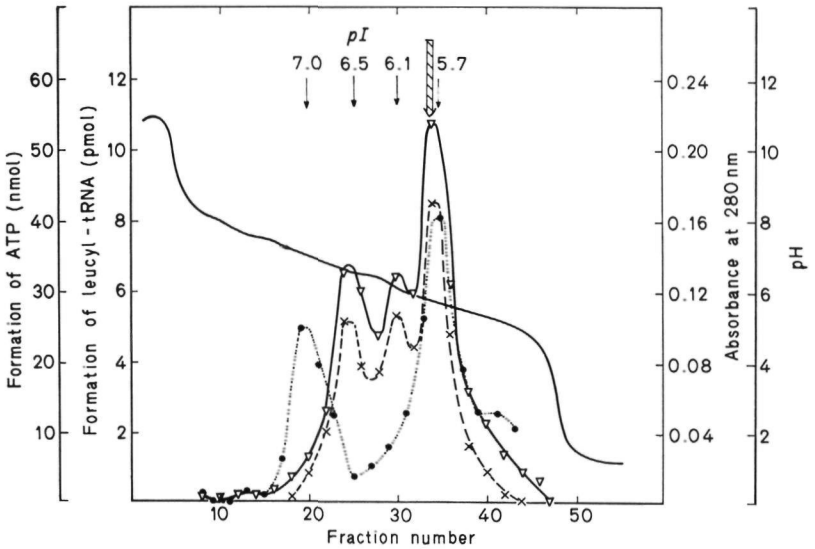


Fig. 5. *Isoelectric focusing of leucyl-tRNA synthetase of fraction X.* Fraction X was purified as described in Fig. 4; 1.6 mg protein in 10 ml was subjected to isoelectric focusing. Synthetase activity was measured both by leucine-dependent ATP-pyrophosphate exchange and by formation of leucyl-tRNA. For pyrophosphate exchange 100 µl of each fraction was assayed. For formation of leucyl-tRNA 20 µl of each fraction was tested as described in Fig. 3. Stimulation of incorporation of amino acids was assayed after extensive dialysis of the fractions. The incubation mixtures (0.25 ml) contained 0.2 mg of supernatant X protein and 40 µg of polyribosomes. Maximal stimulation of incorporation of [^{14}C]leucine is indicated by the hatched arrow. ▽——▽, formation of ATP; ×----×, formation of leucyl-tRNA; ●·····●, absorbance at 280 nm; ——, pH at 0 °C

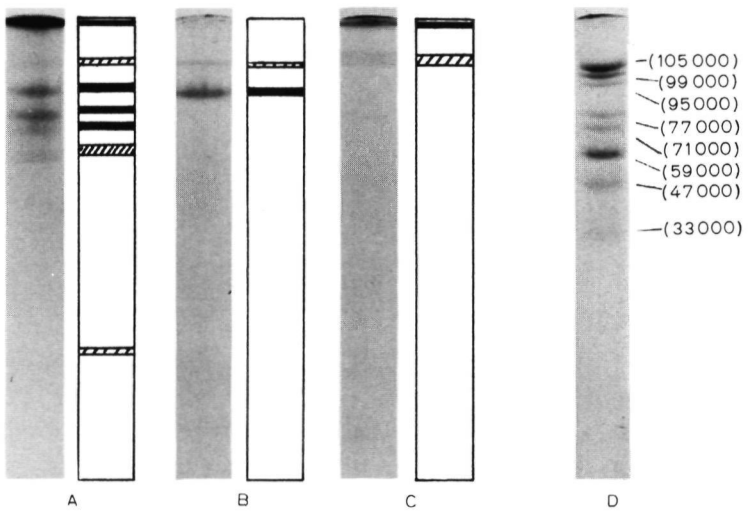


Fig. 6. *Electrophoresis of fraction X on polyacrylamide gels.* (A, B, C) Electrophoresis in 4.5% acrylamide gels at pH 8.9; (D) electrophoresis in a polyacrylamide gel containing sodium dodecylsulphate; (A) active fraction after gel filtration on Sephadex G-200; (B) active fraction after chromatography on DEAE-Sephadex A-50; (C, D) active fraction after chromatography on hydroxyapatite. Estimated molecular weights are given in parentheses

jected to electrophoresis in the presence of sodium dodecylsulphate.

Electron Microscopy

Electron microscopic studies of proteins which play a role in protein biosynthesis are scarce. Shelton *et al.* [49] envisaged the possibility that transferase I exists as a particle which sediments at 19 S. When investigated in the electron microscope, our purified enzyme preparations revealed particle-like structures with a rather well-defined shape.

Fig. 7 A shows the result after negative staining with potassium phosphotungstate. This particle

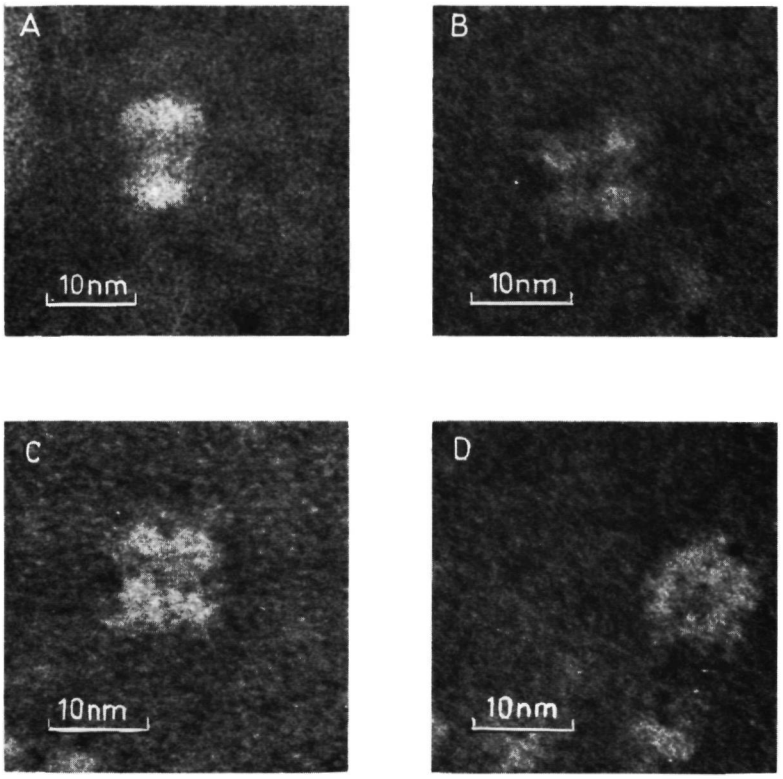


Fig. 7. *Electron micrographs of the active protein-peak after purification.* (A) Protein fraction after chromatography on DEAE-Sephadex A-50. Note the dumb-bell like feature. (B, C, D) Protein fraction after centrifugation in an isokinetic sucrose gradient. Note the distinct globular subunit structure in (B). Negative staining was performed with potassium phosphotungstate (A) or uranyl formate (B, C, D)

resembles those with transferase I activity [49], but is somewhat smaller, approximately 9×12.5 nm. The particle frequently showed a dumb-bell-like feature. After centrifugation of the purified synthetases in a sucrose gradient particle-like structures could also be observed in the electron microscope (Fig. 7 B—D). The particles could only be detected in fractions corresponding to number 18 to 25 in Fig. 4, where both stimulation of incorporation of amino acids and activity of all five aminoacyl-tRNA synthetases had been demonstrated. Two types of particles were observed. One type seemed clearly to be built up by distinct globular subunits (Fig. 7 B). These structures occurred more frequently in the slower sedimenting part of the major protein peak (Fig. 4, fraction 18 to 21). The other type of particles revealed a more compact structure (Fig. 7 C and D) and was mainly found in the faster sedimenting part of the protein peak (Fig. 4, fraction 21 to 25). The rectangular structure in Fig. 7 C and the circular structure in Fig. 7 D presumably represents the same particle viewed from axes perpendicular to each other. In fractions corresponding to numbers 5 to 15 (Fig. 4) no particle-like material was observed.

CONCLUSIONS

Our results enable a more direct interpretation of the action of fraction X. Under the assay conditions used, the activity of this fraction seems to be based mainly upon its content of certain amino-acid-activating enzymes, namely glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase. This can be deduced firstly from our finding that these enzymes were only present in minor quantities in supernatant X. Secondly from the capacity to stimulate incorporation of amino acids, which was always paralleled with the presence of the five synthetases in all purification steps. Thirdly from the observation that the effect of fraction X could only be measured when the incubation mixture contained free amino acids instead of aminoacyl-tRNA [6].

The stimulation achieved with the recombined supernatant X and purified fraction X was of the same order of magnitude as that obtained with supernatant X and crude fraction X. This indicates that the purified fraction still contained all active factors present before purification.

The sedimentation coefficient (18 S) of the purified enzymes was much higher than expected. As

particles with an organized structure were observed in the electron microscope, the results suggest a particle character of the five aminoacyl-tRNA synthetases.

Crude fraction X (Fig. 1) or partially purified fraction X (*cf.* [6]) showed a higher sedimentation value than more purified fraction X (Fig. 4). This probably results from aggregation of the distinct particles as was frequently observed by us. During further purification disaggregation seems to occur into separate particles.

In a recent paper evidence has been provided that in rat liver cells all aminoacyl-tRNA synthetases are integrated into a high molecular weight complex [50]. The authors state that this complex is not an artifact arising during the isolation procedure of the enzymes. The complex of synthetases is very labile and can even be disrupted by excess of homogenization or by freezing and thawing. Our results revealed that certain amino-acid-activating enzymes are lacking in the high molecular weight fraction after partial purification of fraction X (Table 2). This may suggest that some enzymes are more strongly associated than others.

It cannot definitely be excluded that transferase I is also a constituent of a more organized structure. Shelton *et al.* [49] suggest that transferase I is related with particles which resemble those obtained by us after chromatography on DEAE-Sephadex. However, transferase I seems to be bound less tightly to the complex as can be deduced from the results of Shelton *et al.* and from our earlier finding that supernatant X contains saturating quantities of this enzyme [6].

Possibly also initiation factors form an organized structure as the behaviour of factor M_2 and M_3 [51] and EF_m [52] during gel filtration on Sephadex G-200 suggests a high molecular weight. In our routine test system the concentration of magnesium ions was too high to allow the determination of activity of these factors. As at lower concentrations of magnesium ions (4 to 6 mM) the stimulatory effect was as high as when determined at higher magnesium concentrations (7 to 10 mM), it is not very likely that these factors are responsible for the effect of fraction X.

Taking all data available into consideration, we ascribe the action of fraction X mainly to glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase aggregated into a rather stable complex, which is preserved during gel filtration on Sephadex G-200. chromatography on DEAE-Sephadex A-50 and hydroxyapatite and during centrifugation in sucrose gradients.

The authors are obliged to Dr E. L. Benedetti for helpful discussions and criticism, to Dr A. Stols for the electron micrographs and to Miss J. van Westreenen for skilled technical assistance in part of these studies.

REFERENCES

1. Hoagland, M. B. (1961) *Cold Spring Harbor Symp. Quant. Biol. 26*, 153.
2. Hoagland, M. B. & Askonas, B. (1963) *Proc. Nat. Acad. Sci. U. S. A. 49*, 130.
3. Mizrahi, I. J. (1965) *Biochim. Biophys. Acta, 108*, 419.
4. Beard, N. S. & Armentrout, S. A. (1967) *Proc. Nat. Acad. Sci. U. S. A. 58*, 750.
5. Parthier, B. & Hultin, T. (1967) *Biochim. Biophys. Acta, 149*, 562.
6. Vennegoor, C. & Bloemendal, H. (1970) *Eur. J. Biochem. 15*, 161–170.
7. Levin, O. (1962) *Methods Enzymol. 5*, 27.
8. Bloemendal, H., Bont, W. S., de Vries, M. & Benedetti, E. L. (1967) *Biochem. J. 103*, 177.
9. Brunngraber, E. F. (1962) *Biochem. Biophys. Res. Commun. 8*, 1.
10. Konings, R. N. H. (1969) Thesis, Gebr. Janssen, Nijmegen, p. 51.
11. Stulberg, M. P. & Novelli, G. D. (1967) *Methods Enzymol. 5*, 703.
12. Lemoine, F., Waller, J. P. & van Rapenbusch, R. (1968) *Eur. J. Biochem. 4*, 213–221.
13. Mehler, A. H. & Stern, R. (1966) in *Procedures in Nucleic Acid Research*, p. 414, Harper and Row, New York and London.
14. Muench, K. H. & Berg, P. (1966) in *Procedures in Nucleic Acid Research*, p. 375, Harper and Row, New York and London.
15. Heppel, L. A. (1955) *Methods Enzymol. 2*, 570.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
17. Gornall, T. W., Lansford, E. M., Jr & Shive, W. (1949) *J. Biol. Chem. 237*, 751.
18. Bennet, T. P. (1967) *Nature (London), 213*, 1131.
19. Warburg, O. & Christian, W. (1941) *Biochem. Z. 310*, 381.
20. McCarty, K. S., Stafford, D. & Brown, O. (1968) *Anal. Biochem. 24*, 314.
21. Noll, H. (1969) in *Techniques in Protein Biosynthesis.* vol. 2, p. 101, Academic Press, London and New York.
22. Surguchev, A. P., Surgucheva, I. G., Ermokhina, T. M. & Zaitseva, G. N. (1970) *Biochim. Biophys. Acta, 224*, 623.
23. Bloemendal, H. (1963) in *Zone Electrophoresis in Blocks and Columns*, Elsevier, Amsterdam.
24. Shapiro, A. L., Viñuela, E. & Maizel, J. V., Jr (1967) *Biochem. Biophys. Res. Commun. 28*, 815.
25. Weber, K. & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
26. Mellema, J. L., van Bruggen, E. F. J. & Gruber, M. (1968) *J. Mol. Biol. 31*, 75.
27. Leberman, R. (1965) *J. Mol. Biol. 13*, 606.
28. Lewin, B. M. (1970) *The Molecular Basis of Gene Expression*, p. 203, Interscience, London.
29. Stern, R. & Mehler, A. H. (1965) *Biochem. Z. 342*, 400.
30. Baldwin, A. N. & Berg, P. (1966) *J. Biol. Chem. 241*, 831.
31. Vanhumbeek, J. & Lurquin, P. (1969) *Eur. J. Biochem. 10*, 213–218.
32. Stulberg, M. P. (1967) *J. Biol. Chem. 242*, 1060.
33. Calendar, R. & Berg, P. (1966) *Biochemistry, 5*, 1681.
34. Lee, M. L. & Muench, K. H. (1969) *J. Biol. Chem. 244*, 223.
35. Yaniv, M. & Gros, F. (1969) *J. Mol. Biol. 44*, 1.
36. Lazzarini, R. A. & Mehler, A. H. (1966) in *Procedures in Nucleic Acid Research*, p. 409, Harper and Row, New York and London.
37. Ravel, J. M., Wang, S.-F., Heinemeyer, C. & Shive, W. (1965) *J. Biol. Chem. 240*, 432.
38. Deutscher, M. P. (1967) *J. Biol. Chem. 242*, 1132.

39 Deutscher, M P (1967) *J Biol Chem 242*, 1123
40 Fasiolo, F , Befort, N , Boulanger, Y & Ebel J P (1970) *Biochim Biophys Acta, 217*, 305
41 Novelli, G D (1967) *Annu Rev Biochem 36*, 449
42 Cassio, D & Waller, J P (1968) *Eur J Biochem 5*, 33–41
43 Kosakowski, M H J E & Bock, A (1970) *Eur J. Biochem 12*, 67–73
44 Letendre, C H , Humphreys, J M & Grunberg Manago, M (1969) *Biochim Biophys Acta, 186*, 46
45 Lanks, K W , Sciscenti, J , Weinstein, I B & Cantor, C R (1971) *J Biol Chem 246*, 3494
46 Lemaire, G , van Rapenbusch, R , Gros, C & Labouesse, B (1969) *Eur J Biochem 10*, 336–344
47 Preddie, E C (1969) *J Biol Chem 244*, 3958.
48. Vennegoor, C J G M , Stols, A L. H & Bloemendal, H (1972) *J Mol Biol 65* (in press).
49 Shelton, E , Kuff, E L , Maxwell, E S & Harrington, J T (1970) *J Cell Biol 15*, 1.
50 Bandyopadhyay, A K & Deutscher, M P (1971) *J. Mol Biol 60*, 113
51 Prichard, P M , Gilbert, J M , Shafritz, D A & Anderson, W F (1970) *Nature (London), 226*, 511
52 Heywood, S M (1970) *Proc Nat Acad Sci U S A. 67*, 1782

C Vennegoor and H Bloemendal
Laboratorium voor Biochemie der Universiteit
Geert Groteplein Noord 21, Nijmegen

While this paper was in press, two other reports came to our knowledge which have not yet been quoted here

The first paper, entitled 'Isolation from Rat Liver of All Aminoacyl-tRNA Synthetases by Centrifugation', by Geels, J , Bont, W S , and Rezelman, G , appeared in *Arch Biochem Biophys* 144 (1971) 773 The authors determined the activity of 19 amino acid-activating enzymes in a 150 000 *g* pellet and in its supernatant Their results resemble those presented in Table 1 of our paper Moreover, the authors measured the sedimentation coefficient of leucyl-tRNA synthetase of 20 S In the 20 S fraction the specific activity of most aminoacyl-tRNA synthetases was higher than in the 150 000 *g* pellet

A second paper, entitled 'Particulate forms of phenylalanyl-tRNA Synthetase from Ehrlich Ascites Cells', by Roberts, W K , and Coleman, W H , has been published in *Biochem Biophys Res Commun* 46 (1972) 206 Phenylalanyl-tRNA synthetase activity was found to occur in two principle forms activity bound to the ribosomes and activity present as part of a complex sedimenting at approximately 25 S The synthetase activity associated to the ribosomes was removed from the ribosomes after addition of RNA, while concomitantly activity appeared in a complex sedimenting at 25 S.

SUMMARY

This thesis contains an introductory part, a short survey of the process of protein biosynthesis in eukaryotic cytoplasm, a survey of the effect of a post-microsomal fraction on the incorporation of amino acids into ribosomes *in vitro*, and two published papers.

The first paper describes some effects of a post-microsomal fraction, called X fraction, which was isolated from rat liver. In the cell-free system containing polyribosomes, energy and cofactors, the addition of cell sap devoid of the X fraction resulted in a low incorporation of amino acids. When this mixture was supplemented with the X fraction, the incorporation of amino acids into protein was restored to values obtained when an unfractionated post-microsomal supernatant was used as source of soluble enzymes. This effect was not due to either messenger RNA, a ribonuclease inhibitor, or an aminoacyl-tRNA binding enzyme. Stimulation of incorporation of amino acids into protein was not observed when aminoacylated tRNA was added to the incubation mixture and at the same time the action of aminoacyl-tRNA synthetases was prevented. Therefore we concluded that aminoacyl-tRNA synthetases might be responsible for the observed effect.

In the second paper attention is paid to the aminoacyl-tRNA synthetases in the X fraction. At least five of these enzymes were more concentrated in the post-microsomal pellet than in its supernatant. The enzymes were further purified by gel filtration on Sephadex G-200 and chromatography on DEAE-Sephadex A-50 and hydroxyapatite. The purification was 120-170 fold, when compared with the unfractionated post-microsomal supernatant. However, in all these steps no separation of the five aminoacyl-tRNA synthetases occurred. This phenomenon, combined with the unexpected high sedimentation values, led us to the conclusion that the enzymes formed high molecular weight complexes. Transfer RNA was not responsible for the formation of these complexes, since removal of RNA did not result in deaggregation of the enzymes. Electron micrographs revealed particles of well defined size and shape. From these findings we suggested that in rat liver cells at least part of the aminoacyl-tRNA synthetases occur as particles.

SAMENVATTING

Dit proefschrift omvat een inleiding, een kort literatuuroverzicht over het proces van de eiwitbiosynthese in the cytoplasma van eukaryoten, een literatuuroverzicht over het effect van een post-microsomale fractie op de aminozuurincorporatie in ribosomen *in vitro* en twee publicaties.

In de eerste publicatie worden enkele effecten beschreven van een post-microsomale fractie, X fractie genaamd, die uit rattelever werd geïsoleerd. Wanneer celsap waarin de X fractie ontbrak werd toegevoegd aan een celvrij systeem dat polyribosomen, energie en cofactoren bevatte, werd een lage incorporatie van aminozuren verkregen. Na toevoeging van de X fractie aan dit mengsel werd de aminozuurincorporatie hersteld tot waarden die ook werden verkregen wanneer een niet-gefractioneerde post-ribosomale supernatant werd toegevoegd als bron van oplosbare enzymen. Dit effect werd niet veroorzaakt door boodschapper RNA, en evenmin door de aanwezigheid van een ribonuclease-remmer of het aminoacyl-tRNA bindingsenzym transferase I. Wanneer aminoacyl-tRNA werd toegevoegd aan het incubatiemengsel en tegelijkertijd de werking van aminoacyl-tRNA synthetases werd verhinderd, werd geen stimulatie van aminozuurincorporatie door toevoeging van de X fractie waargenomen. Hieruit concludeerden wij dat aminoacyl-tRNA synthetases grotendeels verantwoordelijk zijn voor de werking van de X fractie.

In de tweede publicatie wordt de aandacht gericht op aminoacyl-tRNA synthetases in de X fractie. Tenminste vijf synthetases werden geconcentreerd in het post-microsomale sediment. Deze enzymen werden verder gezuiverd door gelfiltratie op Sephadex G-200 en chromatografie op DEAE-Sephadex A-50 en hydroxyapatiet. De zuivering was 120-170voudig vergeleken met de ongefractioneerde post-microsomale supernatant. Het bleek echter niet mogelijk om de vijf aminoacyl-tRNA synthetases van elkaar te scheiden. Dit verschijnsel en de onverwacht hoge sedimentatiewaarden brachten ons tot de conclusie dat de enzymen waren geaggregeerd tot een hoog moleculair complex. Dit complex werd niet veroorzaakt door binding van tRNA, omdat RNA kon worden verwijderd zonder dat de enzymen desaggregeerden. Op elektronenmicroscopische foto's waren partikeltjes te zien van een gedefinieerde vorm en grootte. Uitgaande van deze resultaten stellen wij voor dat in rattelevercellen tenminste een deel van de aminoacyl-tRNA synthetases als deeltjes aanwezig is.

STELLINGEN

1. De door Sarkar en Moore uitgevoerde groepering van virusachtige partikels in humane melk is aan kritiek onderhevig.

   Sarkar, N.H. & Moore, D.H. On the possibility of a human breast cancer virus. *Nature*, 236 (1972) 103.

2. In de door Faiferman en medewerkers uitgevoerde experimenten wordt niet voldoende rekening gehouden met de mogelijkheid dat de waargenomen verschijnselen kunnen worden verklaard door aspecifieke binding van polysomen aan membranen.

   Faiferman, I., Cornudella, L. & Pogo, A.O. Messenger RNA nuclear particles and their attachment to cytoplasmic membranes in Krebs tumour cells. *Nature New Biology*, 233 (1971) 234.

3. De waargenomen significante verhoging van specifieke immunologische afweer, verkregen na injectie van met neuraminidase behandelde tumorcellen in dieren, maakt het zeer gewenst na te gaan of een therapie door injectie van met neuraminidase behandelde tumorcellen in de mens mogelijk is.

   Bekesi, J.G., St.-Arneault, G. & Holland, J.F. Increase of leukemia L 1210 immunogenicity by *Vibrio cholerae* neuraminidase treatment. *Cancer Res.* 31 (1971) 2130.

   Simmons, A.L., Rios, A., Ray, P.K. & Lundgren, G. Effect of neuraminidase on growth of a 3-methylcholanthrene-induced fibrosarcoma in normal and immunosuppressed syngeneic mice. *J. Nat. Cancer Inst.* 47 (1971) 1087.

   Currie, G.A. Masking of antigens on the Landschütz ascites tumour. *The lancet*, dec. 23 (1967) 1336.

4. Automatisering van de Rimini-Schryver bepaling van allantoïne en zijn afbraakproducten is overbodig indien men de incubaties uitvoert onder meer optimale omstandigheden.

   Pentz, E.I. Adaptation of the Rimini-Schryver reaction for measurement of allantoin in urine. *Anal. Biochem.* 27 (1969) 333.

   Vogels, G.D. & van der Drift, C. Differential analyses of glyoxylate derivatives. *Anal. Biochem.* 33 (1970) 143.

5. Het is te betreuren dat in de door Linskens beschreven experimenten over de invloed van ontgassing van lava op de primaire kolonisering door organismen op het eiland Surtsey een onderzoek naar de startfase van bakteriën, blauwwieren en diatomeeën ontbreekt.

   Linskens, H.F. Influence of degassing from lava on the primary colonization by organisms: a hypothesis. *Nasa Technical Memorandum* X-62, 009, p. 104 (1971).

6. Schimmels vertonen in het verloop van de mitose en in de eigenschappen van de histonen zodanige afwijkingen, dat men deze organismen als een zeer aparte groep onder de eukaryoten zou kunnen plaatsen.

Burnett, J.H. Nuclear division; in: Fundamentals of mycology, p. 360. Ed. Edward Arnold. William Clowes & Sons, London 1968.

Robinow, C.F. & Bakerspiegel, A. Somatic nuclei and forms of mitosis in fungi; in: The fungi, vol. I, p. 119. Eds. G.C. Ainsworth & A.S. Sussman. Academic Press, New York & London 1965.

van der Vliet, P.Ch. De invloed van chromosomale eiwitten op de genexpressie bij gist. Proefschrift. Elinkwijk, Utrecht 1971.

Tonino, G.J.M. & Rozijn, Th.H. On the Occurrence of histones in *yeast*. *Biochim. Biophys. Acta*, 124 (1966) 427.

Dwivedi, R.S., Dutta, S.K. & Bloch, D.P. Isolation and characterization of chromatin from *Neurospora crassa*. *J. Cell Biol.* 43 (1969) 51.

7. De opmerking van van den Ende en van Oorschot, dat '*Ulva lactuca* (als epiphyt) zich met behulp van zijn hapteren tegen de oppervlakte van zijn dragerplant *Himanthalia elongata* vastlegt' en dat 'men slechts af en toe ziet dat door een uiteenschuiven van de bovenste cellagen een betere mechanische verankering wordt verkregen', is in strijd met hun waarneming, dat *Ulva lactuca* op afstervende exemplaren van *Himanthalia elongata* sterk op de voorgrond treedt.

van den Ende, G. & van Oorschot, R. Weitere Beobachtungen über den Epiphytenbewuchs von *Himanthalia elongata* (L.) S.F. Gray. *Bot. Mar.* 5 (1963) 111.

8. De veronderstelling dat een overmaat pH 5 fractie, die is bevrijd van de X fractie, in het incubatiemengsel een significante stimulering door aminozuuractiverende enzymen uitsluit, is onjuist.

Beard, N.S. & Armentrout, S.A. Protein synthesis by reticulocyte ribosomes, III. Description of a ribonucleoprotein fraction which stimulates messenger RNA-ribosomal interaction. *Proc. Nat. Acad. Sci., U.S.A.* 58 (1967) 750.

9. De wijze waarop Spector en medewerkers molecuulgewichtbereiken voor populaties van α-crystallinemoleculen opgeven is voor kritiek vatbaar.

Spector, A., Li, L.-K., Meretsky, D. & Augusteyn, R. What is alpha crystallin? *Amer. J. Ophthalm.* 71 (1971) 386.

10. De conclusie van Cottrell en Avers, dat in gesynchroniseerde cellen de mitochondriale DNA synthese synchroon verloopt, is aanvechtbaar.

Cottrell, S.F. & Avers, C.J. Evidence of mitochondrial synchrony in synchronous cell cultures of yeast. *Biochem. Biophys. Res. Commun.* 38 (1970) 973.